SYNERGIC GAS PRODUCTION BY BACTERIA

by

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INTRODUCTION.

Synergism was suggested by Holman and Meekison (1926) as a suitable term to apply to those reactions in which two or more different types of bacteria growing together in a medium produce by their combined action an end-product which is never found in pure cultures of any one of the associated organisms. On account of their common occurrence synergic reactions resulting in the formation of gas have received most attention. The first systematic work was carried out by Sears and Putnam (1923), who demonstrated synergic gas production by many different pairs of organisms, and found that such pairs always consisted of (1) an organism capable of forming acid from the food substance being tested, and (2) an organism which, whilst unable to produce gas from the test substance, did produce gas from glucose. Further, they concluded that both organisms must be living together in close association in the medium in order to produce gas.

Holman and Meekison (1926) confirmed these results and offered an explanation of the phenomenon in the hypothesis that an intermediate product resulting from the splitting of the test substance by the acid-former becomes available for the other member of the pair, which in turn splits the product so as to form gas. This intermediate substance is available to both bacteria in such a way that the amount of gas formed from it depends on the relative numbers of actively-growing organisms of each type present at the time of its production. Castellani (1928) suggested a similar explanation. He showed that, though *B. Morgani* and *B. typhosus* together formed gas from maltose, the addition of *B. Morgani* to a maltose culture of *B. typhosus* 24 hours old did not result in gas; whereas gas was formed if a younger culture of *B. typhosus* was used. From this he concluded that the decomposition of the sugar by *B. typhosus* has proceeded too far in 24 hours, and no intermediate substance is left to act as a source of gas. Recently this experiment has been repeated by Kossovitch (1933), using sterile filtrates of cultures of *B. typhosus* of varying ages to which *B. Morgani* was added. Gas only appeared in filtrates of cultures 5 to 8 hours old. This result supports Castellani's views.

In spite of all this evidence Graham (1932) showed that *B. typhosus* can produce from mannitol a stable intermediate substance which *B. Morgani* can further degrade to gas. He killed cultures of *B. typhosus* in mannitol when they were 24, 48, and 72 hours old, respectively, and inoculated them with *B. Morgani*, obtaining gas in every case, the greatest volume corresponding to the *B. typhosus* culture with the longest incubation period. Analysis of the synergic gas showed a much larger ratio of hydrogen to carbon-dioxide than that found in the gas formed from glucose by the glucose-fermenter, while this high ratio was similar to that obtained in the gas from the fermentation of formates by this organism. Harden (1930) and Castellani (1928) had both suggested formic acid as a possible precursor of synergic gas, and Graham's results add confirmation to this idea.

The conflicting opinions of the various authors quoted indicate the need for further investigation of the whole subject. The present work offers more evidence for the presence of a stable intermediate substance in cultures of *B. typhosus*, and is of special interest as it shows synergic gas production from a pentose, viz. xylose. The last part of the paper, in which tests with streptococci are recorded, is in the nature of a progress report as experiments are still proceeding.

I. SYNERGIC GAS PRODUCTION BY *B. TYPHOSUS* and *B. MORGANI*.

a. Experiments with Xylose.

Synergic reactions involving pentoses have not been investigated previously though suitable pairs of organisms can easily be found. For example, *B. typhosus* forms acid from xylose, which *B. Morgani* does not attack, although it forms acid and gas from glucose. According, therefore, to Sears and Putnam's general description of a synergic pair, these two bacteria together should form gas from xylose. On testing this out the writer obtained a positive result, and the action of this pair on xylose was chosen for closer study. Having demonstrated synergic gas production when both organisms were living together, preliminary tests were made to test for the presence of a stable intermediate substance in the xylose culture of *B. typhosus*.

This organism was therefore grown in 1 p.c. xylose-peptone-water cultures provided with Durham tubes. The cultures were killed by heat after 48 hours incubation, and tested for sterility. *B. Morgani* was then added, and a large bubble of gas appeared after 2 days' further incubation. As cultural tests had shown the *B. typhosus* cultures to be sterile, it was concluded that the gas must have come from some intermediate substance occurring in small amount in these dead typhoid cultures.

Tests were therefore continued, using U-shaped fermentation tubes in which the volume of gas developing from the medium in the closed arm could be measured fairly accurately. As all the tubes contained the same amount of culture medium in this closed arm, the gas volumes measured in this way could be used for comparison under varying conditions. 1 p.c. xylose-peptone-water was used in all the tests. In Graham's work a longer incubation period had re-

sulted in the production of more intermediate substance, and therefore the first batch of fermentation tubes sown with *B. typhosus* was incubated for 5 days. The contents of these tubes were then neutralized, the organisms killed by heat, and after sterility tests had been made, were sown with *B. Morgani*. The results were disappointing, as some tubes showed no gas at all, while others showed only 0·1 c.c. Thus in some cases the intermediate substance seemed to be present, but its quantity was very small.

If the continued growth of *B. typhosus* be necessary to produce more and more of the stable intermediate substance, a long incubation period alone will not bring this about, because the acidity, which develops very early, rapidly becomes inhibitory; but if this acid can be neutralized as it is formed then the organism could continue to grow for a much longer time with the continued production of the intermediate substance. Calcium carbonate has often been used successfully to check the development of undue acidity in culture media, and it was therefore now added to the medium in which *B. typhosus* was to be grown.

To determine the effectiveness of this device, parallel cultures of *B typhosus* were made *with* and *without* calcium carbonate, each series including an uninoculated control (i.e. *without B. typhosus*). After five days' incubation, sterile calcium carbonate was added in excess to the series without it, to neutralize the acidity, and all cultures were killed by heat, their sterility being established by cultural tests. The cultures so treated were distributed in fermentation tubes, and *B. Morgani* added to all.

After three days' incubation some gas was visible in those tubes from the *B. typhosus* cultures without calcium carbonate, but, as in the previous test, the volume was very small, usually 0·1 c.c. or less.

The result was quite different, however, in the tubes from *B. typhosus* cultures with calcium carbonate, for here gas production was comparatively good, amounting in some cases to 2·3 c.c. and generally not less than 2 c.c. None of the uninoculated controls gave any gas when subsequently sown with *B. Morgani*, showing that the amount of heat used to kill *B. typhosus* could not have caused any decomposition of the xylose into a form fermentable by *B. Morgani*. As an extra control on the sterility of the *B. typhosus* cultures, fresh xylose-peptone-water tubes were sown from every gas positive tube, and invariably showed neither acid nor gas, but only a turbidity due to the growth of *B. Morgani*.

A similar type of experiment was next planned using culture filtrates instead of simply dead cultures. *B. typhosus* was grown for five days in xylose-peptone-water, with and without calcium carbonate; any acidity in the tubes without carbonate was then neutralized by its addition, and all the cultures were filtered through a Seitz (EK) bacterial filter. The filtrates, after sterility tests had been made, were placed in fermentation tubes and sown with *B. Morgani*.

The amounts of gas obtained were very like those resulting from dead cultures, but on the whole the volumes were slightly less. Thus no gas or only a large bubble was formed in filtrates of cultures without calcium carbonate, but 2 c.c. of gas were regularly formed from filtrates of cultures with calcium carbonate. No gas was obtained from filtrates of uninoculated control cultures.

When *B. typhosus* and *B. Morgani* were sown together into the same medium under the same conditions as the experiments given above, gas production was variable, but the maximum volume was 1·8 c.c. It has therefore been possible by the use of calcium carbonate in the cultures of *B. typhosus* to obtain more gas by the growth of *B. Morgani* in dead cultures (2 c.c.) than the maximum volume given by the combination of the two organisms growing together (1·8 c.c.). There thus seems to be no doubt of the facilitating effect of calcium carbonate on the production of the stable precursor of gas by *B. typhosus* in xylose medium, and as heat-killed cultures as well as filtrates were used in the tests this substance must be fairly heat-stable.

The success of the introduction of calcium carbonate in cultures of *B. typhosus* led to a trial of its effect when *B. Morgani* and *B. typhosus* were growing together in xylose medium.

Both organisms were sown together into two sets of fermentation tubes, identical except for the presence of calcium carbonate in one set. In the absence of calcium carbonate, gas production was irregular, the maximum volume being 1·8 c.c., but, in the presence of calcium carbonate, gas was constantly formed in volumes of 4·5 c.c. for each tube, so that the gas occupied almost half the closed arm. Small variations in the relative size of the inoculum for each organism, though affecting the amount of gas formed in the absence of calcium carbonate, always resulted in the same amount of gas (i.e. 4·5 c.c.) when calcium carbonate was present.

This increase in volume is not due to any carbon dioxide evolved from the calcium carbonate during neutralization, for even when calcium carbonate has been used to neutralize the total acidity produced by *B. typhosus* during five days' incubation, the amount of carbon-dioxide resulting is negligible in volume. The presence of calcium carbonate in the mixed culture has therefore made possible the production of a very much larger volume of gas.

The role of calcium carbonate seems to be simply that of a neutralizing agent, as suggested by the following tests. Cultures of *B. typhosus* were made in xylose-peptone-water in flasks, with and without calcium carbonate, and the acidity in those without calcium carbonate was neutralized night and morning with sterile soda solution. After five days' incubation a portion of the contents of each flask was filtered through a Seitz (EK) filter, and the remainder sterilized by heat. The filtrates and dead cultures were placed in fermentation tubes, and sown with *B. Morgani*. Approximately 2 c.c. of gas developed in all tubes, irrespective of whether their contents had been neutralized by soda or grown in the presence of calcium carbonate. Thus the neutralizing action seems to be the main factor in increasing the yield of intermediate substance.

b. Experiments with Mannitol.

Some of Graham's (1931) experiments were repeated to test the effect of calcium carbonate on the production of the intermediate substance from mannitol. Filtered and heat-killed cultures of *B. typhosus* in 1 p.c. mannitol-peptone-water, with and without calcium carbonate, were tested with *B. Morgani* for gas

production. The results were similar to those given with xylose, for much larger volumes of gas were obtained from filtered or heat-killed typhoid cultures previously grown with calcium carbonate. On the whole more gas was obtained from mannitol than from xylose in this way.

B. typhosus thus produces from both xylose and mannitol a stable intermediate substance which *B. Morgani* can degrade to gas, and the amount of this substance is greatly increased in both cases by growth in the presence of calcium carbonate. The reason for this appears to be in the neutralizing effect of the calcium carbonate on the acids produced by *B. typhosus*, with the result that this organism remains longer in an actively-growing state and continues to decompose the carbohydrate.

II. SYNERGIC GAS PRODUCTION BY *B. MORGANI* AND VARIOUS STREPTOCOCCI.

It has been suggested, as previously noted, that formic acid is the substance which acts as the precursor of synergic gas. If this view is correct it should be impossible for an organism, which produces acid *but no formic acid* from a test substance, to act as one of a synergic pair, for there would be no intermediate substance formed for the second organism to decompose to gas. If such a pair could be found the results of tests undertaken with them should be extremely interesting.

Langwill (1927) has stated that haemolytic streptococci, as opposed to the non-haemolytic or the viridans type of streptococci, do not form formic acid when they decompose carbohydrates. The experiment to be described was therefore commenced by determining the fermentation reactions for a number of haemolytic streptococci, all of which were found to give acid from lactose. *B. Morgani* does not ferment this sugar, though it forms acid and gas from glucose, and therefore this organism and any one of the haemolytic streptococci conform to the requirements of a synergic pair, as defined by Sears and Putnam, but according to Langwill the acid-former does not produce formic acid.

All the streptococci grew well in 1 p.c. lactose-peptone-water, and preliminary tests for synergic gas were made in cultures of this medium containing Durham tubes, and sown simultaneously with the haemolytic streptococci and *B. Morgani*. In every case a large bubble of gas appeared after 48 hours' incubation.

B. Morgani and a haemolytic streptococcus were then sown together into fermentation tubes containing lactose-peptone-water, with and without calcium carbonate. In every case appreciable quantities of gas developed during the first 48 hours' incubation, but the volume seemed to vary somewhat with the different strains of haemolytic streptococci. The effect of the calcium carbonate was not always so marked as with *B. typhosus* and *B. Morgani*, but generally those tubes with calcium carbonate developed approximately twice as much gas as the others. Thus with one pair used in these experiments 0·7 c.c. of gas was generally formed in the absence of calcium carbonate, while 1·8 c.c. was formed when calcium carbonate was present.

Haemolytic streptococci can therefore produce some substance which can act as a precursor of gas, and the reported absence of formic acid in cultures of

these organisms, if correct, leads to the conclusion that in this case at any rate, this substance is not necessary for the formation of gas.

A non-haemolytic streptococcus which, according to Langwill, does produce formic acid, was also tested out with *B. Morgani* in lactose medium. The amount of gas produced by the combined growth of the two organisms was much the same as that produced with the haemolytic streptococci and the effect of calcium carbonate was the same, the maximum volume of gas in presence of calcium carbonate being 1·7 c.c. Here, of course, formic acid may be assumed to be formed and to give rise to the gas, but it is strange that the volume should be so similar with both types of streptococci regardless of the difference in their alleged ability to give formic acid.

Having demonstrated that both haemolytic and non-haemolytic streptococci could with *B. Morgani* give rise to the production of gas, the next step was the testing of these streptococcal cultures for the presence of a stable intermediate substance from which *B. Morgani* could form gas. The organisms were grown in lactose-peptone-water with and without calcium carbonate, and filtrates and heat-killed cultures were sown with *B. Morgani*, the general details of the tests being the same as for *B. typhosus*.

Gas appeared in the dead cultures and filtrates of the non-haemolytic streptococci but not in those of the haemolytic streptococci. With the former, growth in the presence of calcium carbonate had apparently given the larger quantity of the intermediate substance, for the volume of gas obtained from such cultures was 0·6 c.c., while in cultures of the same streptococcus without calcium carbonate the amount of gas was only 0·2 c.c.

Thus, though non-haemolytic streptococci produce a stable intermediate substance, haemolytic streptococci do not appear to do so.

In contrast to this experiment, it is remarkable that a five-days-old culture of haemolytic streptococci incubated with calcium carbonate and left unheated did give rise to 0·5 c.c. or more of gas when sown with *B. Morgani*. It does not seem likely that the amount of intermediate substance necessary to form this volume of gas could have been produced by the few streptococci still growing after five days. It seems more feasible to suppose that this intermediate substance was already there, and yet a killed or filtered haemolytic streptococcus culture identical in other respects with this one will not give gas.

As haemolytic streptococci do give synergic gas from lactose with *B. Morgani*, it seemed advisable to test cultures of various strains in lactose-peptone-water for formic acid. Langwill's (1927) test method was used, in which the culture, acidified with sulphuric acid, was steam distilled, and to the distillate, made alkaline with Na_2CO_3 and heated, was added drop by drop a solution of 1 p.c. $KMnO_4$. Decolourization was taken to indicate the presence of formic acid. In the present case, 1 p.c. $KMnO_4$ was much too strong, and 0·1 p.c. $KMnO_4$ was used; but the amount of decolourization obtained with various haemolytic streptococcus cultures was negligible, seeming to indicate the absence of formic acid. On the whole, however, the method did not seem very satisfactory, and further work is now in progress in which more detailed

analyses of cultures of both haemolytic and non-haemolytic streptococci are being made, and these results will be published later.

For the present it appears that, though haemolytic streptococci are stated not to produce formic acid, and the few tests carried out here support this view, they can form a synergic pair with *B. Morgani* and give gas from lactose.

Thus either haemolytic streptococci do produce formic acid—contrary to Langwill's findings—or else formic acid is not the precursor of gas in this reaction. The suggestion might be made that formic acid is a transitory breakdown product, unable to be waylaid and identified by definite tests, but this is improbable, for the only result of any further breakdown of formic acid must be gas—which does not occur in pure streptococcus cultures.

In the case of the non-haemolytic streptococci, they also are able to produce synergic gas with *B. Morgani* from lactose, but here formic acid has been described by Langwill as one of the products, and moreover our experiments as described herein have proved the presence of a stable intermediate substance in the dead or filtered streptococcus cultures from which *B. Morgani* is able to form gas.

SUMMARY AND CONCLUSIONS.

1. *B. Morgani* and *B. typhosus* together form synergic gas from xylose.

2. There is present in old cultures of *B. typhosus* in xylose medium a stable intermediate substance which *B. Morgani* can degrade to gas.

3. The amount of the intermediate substance is greatly increased by growth of *B. typhosus* in media containing calcium carbonate due to the neutralizing effect which allows continued growth of the organism.

4. Calcium carbonate also causes a great increase in the amount of gas produced when both organisms are growing together.

5. *B. Morgani* in combination with either a haemolytic or a non-haemolytic streptococcus forms synergic gas from lactose.

6. Calcium carbonate has had the same effect with these reactions, and by its use a stable intermediate substance which *B. Morgani* can degrade to gas has been demonstrated in cultures of a non-haemolytic streptococcus.

7. The formation of synergic gas by *B. Morgani* with a haemolytic streptococcus either disproves the view that the latter cannot form formic acid, or leads to the conclusion that here formic acid is not the precursor of the gas.

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THE VOLUMETRIC MICRO-DETERMINATION OF CALCIUM WITH METHYLENE BLUE FOLLOWING ITS PRECIPITATION AS CALCIUM PICROLONATE

by

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PRINCIPLE.

From a neutral or slightly acid solution calcium is precipitated as calcium picrolonate by means of a known excessive amount of a standardized solution of lithium picrolonate. After filtering off the precipitated calcium picrolonate, the excess of picrolonate added is determined on an aliquot part of the filtrate by titration with methylene blue.

REAGENTS.

1. 0·05N lithium picrolonate. 6·6 gm. of picrolonic acid are dissolved in 500 c.c. of a 0·05N solution of lithium carbonate. The solution must be neutral or slightly acid. It is filtered after standing overnight, and is then standardized against methylene blue.
2. 0·01N methylene blue. This is best prepared by dissolving 3·74 gm. of methylene blue, chemically pure (Merck), in water and diluting to a volume of one litre. It is standardized against a picric acid solution of known titre as described in a previous communication. (Bolliger, 1933.)

PROCEDURE.

The solution to be analysed should be neutral or slightly acid in reaction. Alkali salts should not be present in a concentration higher than 0·05N. Barium and heavy metals, if present, are best removed beforehand, but, as will be shown, there is no objection to magnesium being present in considerable amounts.

A measured amount of the standardized lithium picrolonate is added to the solution till a distinct but not large excess has been established. After mixing well the solution is kept in the ice-box for at least five hours. Standing overnight is advisable, inasmuch as the precipitate of calcium picrolonate which is at first very voluminous and finely crystalline, contracts and becomes coarsely crystalline, and eventually a contaminating precipitate of magnesium or alkali

pierolonate may be noted (Robinson and Scott, 1934). The precipitate is then filtered off and immediately afterwards an aliquot part of the filtrate is transferred to a separatory funnel containing chloroform and some calcium carbonate.

The titration of the pierolonic acid present in the filtrate is the same as that described elsewhere for picric acid or other o-nitrophenols (Bolliger, 1933, 1934). The standard methylene blue solution, usually 0·001N, is added from a burette to the solution in the separatory funnel. The methylene blue pierolonate formed is only slightly soluble in water, but is easily extracted by chloroform. The colour of the chloroform solution is green, and the yellow colour of the pierolonic acid solution diminishes to an extent proportionate to the amount of methylene blue added. On account of the comparatively great solubility of methylene blue pierolonate in chloroform, and on account of its small solubility in water, one may at first extract only very superficially by whirling the funnel around, but when the watery layer becomes distinctly greenish-blue, then one extracts more thoroughly and replaces with fresh chloroform at least twice. The end point is reached after the first appearance of an unextractable blue colour in the aqueous layer, and a smaller amount of fresh chloroform added should not turn green after the addition of some more methylene blue and subsequent extraction.

DISCUSSION.

Of all the aromatic o-nitrohydroxy-compounds examined so far as to their suitability for methylene blue titration (Bolliger, 1934), pierolonic acid has been found to be the most useful. The solubility of its methylene blue compound is slight in water but considerable in chloroform. The end point is consequently very sharp, even when dealing with 0·001N solutions. Therefore, this observation was applied to the volumetric determination of calcium as pierolonate, a method which has been previously applied by Dworzak and Reich-Rohrwig (1931) for its gravimetric determination. These authors claim that with their method 2 mg. of calcium can be determined with an accuracy of 0·01 mg., and that an amount of magnesium ten times as great as that of calcium does not interfere. With the volumetric micro method described in this paper, even 0·3 mg. of calcium can be determined with a sufficient degree of accuracy in solutions containing only calcium (table 1, experiment 1). The presence of as much as twelve times magnesium and ammonium as calcium affects the accuracy of the method only slightly (experiments 6, 7). However, as shown in the table, the conditions become less favourable when appreciable amounts of sodium or potassium are present (experiments 8, 9, 10). Alten, Weiland, and Knippenberg (1933), who described a colorimetric method for the determination of calcium as pierolonate were also able to confirm the claims of Dworzak and Reich-Rohrwig. They examined a solution which contained calcium and magnesium and various alkali salts in the following proportion: $Ca : Mg : K : Na : NH_3 = 1 : 2 : 5 : 2 : 5$. They found that in

these proportions alkali salts do not interfere in the presence of 0·1 mg. of calcium or less per cubic centimetre. But as soon as the amount of sodium or potassium as compared with calcium is increased considerably more than two-fold, magnesium may be precipitated if a sufficient excess of lithium picrolonate has been added. In such a case, as shown in experiment 9, magnesium present as magnesia mixture was almost completely precipitated in the presence of sodium in a concentration of 0·07N.

But even when magnesium is either absent or present in only small amounts, the presence of large amounts of alkali salts may interfere on account of the only moderate solubility of the alkali (lithium excepted) picrolonates. If sodium be present in a concentration above 0·02N, precipitation of some sodium picrolonate may be expected (experiment 10). This cannot be avoided by further dilution on account of the still appreciable solubility of calcium picrolonate. Mainly for this reason, the more soluble lithium picrolonate, which can be added in a more concentrated form, was chosen as precipitant. Lithium picrolonate still remains in solution at a concentration of 0·5N, while a 0·02N solution of the sodium salt or a 0·01N solution of the free picrolonic acid is already saturated at room temperature.

Strongly acid solutions cannot, however, be neutralized with lithium carbonate, and most of the acid has to be removed by other means, otherwise the results will be too low. As found experimentally, in an approximately 0·2N solution of lithium sulphate containing 0·029 p.c. calcium, the results were as much as 15 p.c. too low using lithium picrolonate as precipitant. Only when a calcium concentration of about 0·2 p.c. is reached will the loss be below 5 p.c. With sodium salts this effect is also present in those cases where sodium picrolonate is not actually precipitated out. This is apparently the case in the presence of considerable amounts of magnesium (experiment 8).

On microscopical examination of the precipitated picrolonate the presence of alkali picrolonate or magnesium picrolonate may easily be detected in comparison with the typical appearance of the highly refractive crystals of calcium picrolonate. If it is found that sodium picrolonate has probably been precipitated together with the calcium picrolonate the situation may be sometimes remedied by adding sufficient of a known amount of standardized methylene blue solution to re-dissolve the precipitated sodium picrolonate. The sparingly soluble methylene blue picrolonate which is precipitated, is filtered off together with the precipitated calcium picrolonate, and on an aliquot part of the solution the remaining picrolonate is determined. The following precautions, however, have to be observed. The calcium picrolonate has to be precipitated completely, i.e. the mixture has to be kept on ice overnight, and too great a volume of methylene blue solution should not be added, in order to avoid excessive dilution. Then after adding the methylene blue, the sample has to be mixed well and left on ice for about another hour. As shown in the table (experiment 11) it is possible to

analyse an 0·1N solution of sodium chloride containing 0·029 p.c. calcium. The results, although somewhat low, are still useful, and the presence of smaller amounts of magnesium does not interfere.

Recovery of Known Amounts of Calcium.

Exp. No.	$CaCl_2$. mg. Ca.	$MgCl_2NH_4Cl$. mg. Mg.	NaCl. mg. Na.	Meth. blue. c.c.	Concentration Ca. p.c.	Ca. found. mg.	Error. p.c.
1	0·029	—	—	—	0·003	0·028	—0·3
2	0·087	—	—	—	0·01	0·086	—1·2
3	0·29	—	—	—	0·03	0·289	—0·3
4	1·45	—	—	—	0·3	1·45	0
5	0·58	0·21	—	—	0·015	0·572	—1·4
6	0·58	0·525	—	—	0·008	0·571	—1·6
7	1·45	18·0	—	—	0·15	1·48	+2·0
8	0·58	0·315	4·6	—	0·012	0·56	—3·6
9	0·29	0·525	10·0	—		1·5	Mg. precipitated
10	1·45	—	11·5	—		1·55	+7·0
11	1·45	—	11·5	10		1·42	—2·6

It should also be mentioned that the precipitated calcium picrolonate may be titrated with methylene blue after it has been dissolved in a small amount of pyridine. But when dealing with several estimations, the determination of the excess picrolonic acid is the simpler procedure, and in this form the determination of calcium as calcium picrolonate may, in the absence of larger amounts of alkali salts, occasionally be preferred to the still much criticized oxydimetric determination of calcium. Specific examples of its application will be discussed on another occasion.

SUMMARY.

A simple method has been described for the volumetric determination of calcium as calcium picrolonate. With a known excessive amount of lithium picrolonate, calcium is precipitated as picrolonate and the excess lithium picrolonate is titrated with methylene blue. The limitations of the method have been discussed.

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THE ACTION OF MUSSEL POISON ON THE NERVOUS SYSTEM

by

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Outbreaks of paralytic poisoning following the consumption of sea mussels have been recorded in Europe and on the Pacific coast of North America. These records have been summarized by Meyer, Sommer, and Schoenholz (1928), who investigated an extensive outbreak in California in 1927.

The symptoms of poisoning indicate predominant action on the nervous system. Paraesthesias in the mucous membrane of the mouth and in the limbs quickly follow the consumption of poisonous mussels, and the early mental excitement, ataxia, giddiness, and incoherence of speech are reminiscent of acute alcoholism. Paralysis of the skeletal muscles may ensue with death from respiratory failure within twelve hours.

The pharmacology of the poison prepared by Sommer from American mussels has been studied by Prinzmetal, Sommer, and Leake (1932). These workers have shown that it is absorbed from the gastrointestine and rapidly eliminated by the kidneys. They describe its depressant effect upon respiration, large doses causing instantaneous failure and moderate doses causing, first, slowing of the rate of respiration, then dyspnoea with short, violent expirations, and finally gasping and cessation of respiration. The poison also causes a profound fall of blood pressure, the course of which varies in different species of experimental animals. It has no effect upon intestinal or uterine smooth muscle, but impairs conduction in the amphibian heart.

Through the courtesy of Dr. Sommer, of the Hooper Foundation, I have been able to extend these results by a further analysis of the action of the poison. The sample used was prepared by Dr. Sommer from poisonous mussels of Oregon in 1933. It has been kept at room temperature in a thoroughly dried state, and has undergone no appreciable loss of toxicity since it was tested in San Francisco in June, 1934.

Prinzmetal, Sommer, and Leake (1932) have defined a lethal dose as that amount which injected intraperitoneally in white mice of 20 gm. weight causes death in from 10 to 20 minutes. The lethal dose estimated in this way in June, 1934, was 0·1 mg. In January, 1935, ten white

mice which received 0·1 mg. per 20 gm. in a concentration of 0·5 mg. per c.c., all died with typical symptoms in between 8 and 16 minutes.

THE EFFECT OF THE POISON UPON RESPIRATION.

A few preliminary observations were made by the injection of the poison in a concentration of 2·0 mg. per c.c. into the marginal ear veins of unanaesthetized domestic rabbits. The lethal dose was about 1·1 mg. per kg. In Table 1 are set out some typical results.

TABLE 1.

Effects of Intravenous Injection in Rabbits.

Weight in kg.	Dose in mg. per kg.	Result.
2·09	0·6	Respiration rate before injection, 180 per minute; after, in successive minutes, 100, 73, 96, 134.
1·9	0·65	Respiration rate before injection, 180 per minute; after, in successive minutes, 66, 40, 60, 100, 140.
2·02	0·8	Only a few irregular respirations in the first minute. Rate 40 per minute in the second minute, and gradual recovery by 7 minutes.
1·94	1·0	Extremely severe symptoms. In the first minute respiration practically ceased. After three minutes the rate was 30 per minute, and the respirations costal in type. After ten minutes they were costal and strongly dyspnoeic, rate 34 per minute. After 13 minutes the rate was 68 per minute. After 17 minutes the animal had recovered. During the first 5 minutes there were profound shock and loss of tone—asphyxial "starting movements"—and the head lay in a position determined by gravity.
1·98	1·1	Death in 4½ minutes. Failure of respiration in the first minute, during which there were only occasional feeble respirations—no dyspnoea, but collapse and loss of tone, with "starting movements". In the terminal gasps only the mouth and alae nasi were active. Convulsions were absent, and the heart was found beating after death. Faradic stimulation of the phrenic nerves caused no contraction of the diaphragm at 0 cm.; direct stimulation was effective at 26 cm. separation of the primary and secondary coils. The sciatic endings were not curarized, a good response being obtained by indirect stimulation at 40 cm., the thigh muscles responding directly at 22 cm.
2·1	1·2	Death in 2½ minutes with failure of respiration and profound collapse. The phrenic endings were curarized, and the sciatic endings somewhat affected. (Indirect stimulation, 34 cm.; direct, 26 cm.)

Apart from the slowing of respiration following the injection of sublethal doses of poison, a phenomenon which, as will be seen, cannot be accounted for by the simultaneous fall of blood pressure, these observations do not emphasize the direct effect of the poison upon the respiratory centres. They do, however, indicate that with this range of dosage the peripheral action of the poison in causing curarization of the motor endings of the diaphragm may play a part in producing symptoms. In the animal which recovered after receiving 1·0 mg. per kg. the symptoms, apart from the early extreme slowing of the rate of respiration, were suggestive of peripheral respiratory failure by curarization, and in animals which died, curarization of the diaphragm was demonstrated at death.

With somewhat larger doses, in animals kept alive under anaesthesia by artificial respiration, the whole skeletal musculature was curarized. Such animals could be kept alive under artificial respiration despite the repeated administration of large doses of poison given at sufficient intervals to allow of the recovery of blood pressure. Curarization and recovery took place rather more slowly than after repeated doses of curari. Experiment 1 illustrates this type of experiment.

Experiment 1.

A cat weighing 2·29 kg. was anaesthetized with dial 0·15 gm. intraperitoneally. Under additional ether anaesthesia the blood pressure was recorded, a femoral venous cannula tied in, and the left sciatic nerve exposed for stimulation. Faradic stimulation was applied with the primary and secondary coils fixed 20 cm. apart, and the stimulating current was varied by means of a wire resistance a metre long of 13 ohms in the primary circuit (E.M.F. 2 volts).

11.53 a.m. 2·0 mg. mussel poison was injected intravenously.
12.13 p.m. 4·5 mg. mussel poison was given. The respirations failed, and artificial ventilation was applied.
12.30 p.m. Sciatic stimulation with 15 cm. resistance gave a feeble response.
1.22 p.m. Sciatic stimulation with 43 cm. resistance gave a good response.
1.25 p.m. Artificial ventilation was discontinued for a minute, but the animal failed to breathe.
2.13 p.m. Animal breathing naturally.
2.22 p.m. 3·0 mg. mussel poison. Artificial ventilation had to be resumed.
2.31 p.m. 2·0 mg. mussel poison.
2.32 p.m. Sciatic stimulation, feeble response at 27 cm. resistance.
2.38 p.m. 3·0 mg. mussel poison.
2.40 p.m. Curarization complete—no response to stimulation of the sciatic.
6.15 p.m. Curarization still complete.
8.30 p.m. Sciatic stimulation, good response at 37 cm. resistance.
8.35 p.m. Animal breathing naturally.
9.42 p.m. Sciatic stimulation gave a good response at 48 cm. resistance.

Experiments with amphibian nerve muscle preparations later to be recorded confirmed these results and showed that the poison was an extremely potent curarizing agent.

The central action of the poison can best be shown by the intravenous injection of large doses. In intact animals the injection of a dose equivalent to three or more lethal doses causes immediate cessation of all respiratory activity before the blood pressure has fallen to any appreciable extent. Figure 1b shows this effect in a rabbit anaesthetized with dial and ether in which both carotid arteries had been ligated and the vagi divided. In contrast in Figure 1a is shown the effect of a rapid fall of blood pressure caused by cutting across the abdominal aorta in a similarly prepared animal. Here there was an increase in the respiratory excursions followed by a period of gasping respiration with cessation of respiratory movement five minutes after the blood pressure had fallen almost to zero.

It may be noted that ligation of the carotids is not adequate to exclude action through the carotid sinuses. For this it is necessary to denervate the sinuses, or to exclude them from cir-

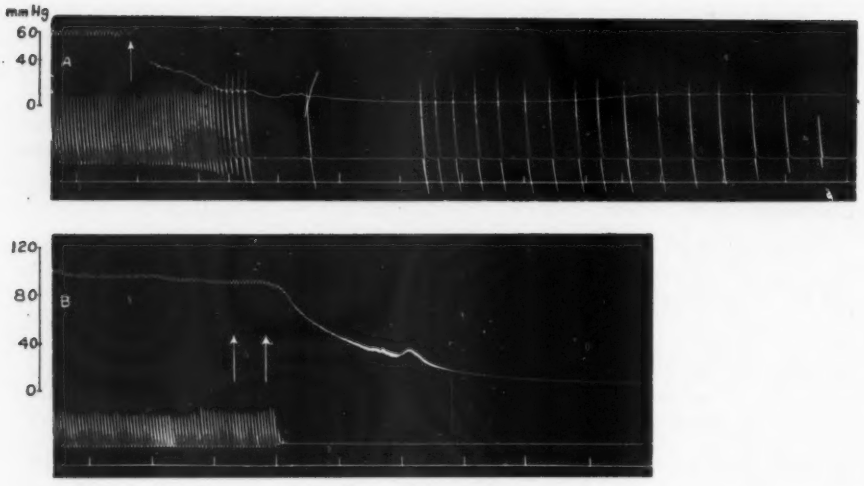

Figure 1. Blood pressure and tracheal respiratory tracings from rabbits under dial (0·1 gm. intraperitoneally) and ether anaesthesia, in which both common carotid arteries were tied and the vagi divided (a) from an animal weighing 1·84 kg. At the arrow, the abdominal aorta was divided transversely; (b) from an animal weighing 2·05 kg. Between the arrows 4·1 mg. of mussel poison was injected intravenously. Time in half minutes.

culation by ligation of the common carotids and of all their branches distal to the sinuses. Animals so prepared, in accord with Schmidt's observations (1932), give no immediate respiratory response to anoxaemia but still respond by increased lung ventilations to sudden fall of blood pressure.

The effects upon respiration of mussel poison in these large doses are precisely similar in intact and in completely "unbuffered" animals. This can only indicate a primary and profoundly depressant action of the poison upon the respiratory centres.

In rabbits anaesthetized by the intraperitoneal injection of dial (50 mg. per kg.) with additional ether for the operative procedures, a dose of from 0·5 to 0·7 mg. per kg. of mussel poison after the ether has been eliminated causes failure of respiration, generally without curarization of the diaphragm being detectable at death. The central depressant action of the poison apparently sums with the depression due to the barbituric acid derivative. In these experiments the changes in the descending action currents in the fourth root of the phrenic nerve were studied by amplification and optical record with the Matthews oscillograph, together with a tracheal record of the respiratory movements. Figure 2 illustrates this type of experiment in which less than 0·5 mg. per kg. of mussel poison caused death in 4½ minutes without any detectable curarization. The oscillograph record, however, indicates that although curarization could not be demonstrated by fara-

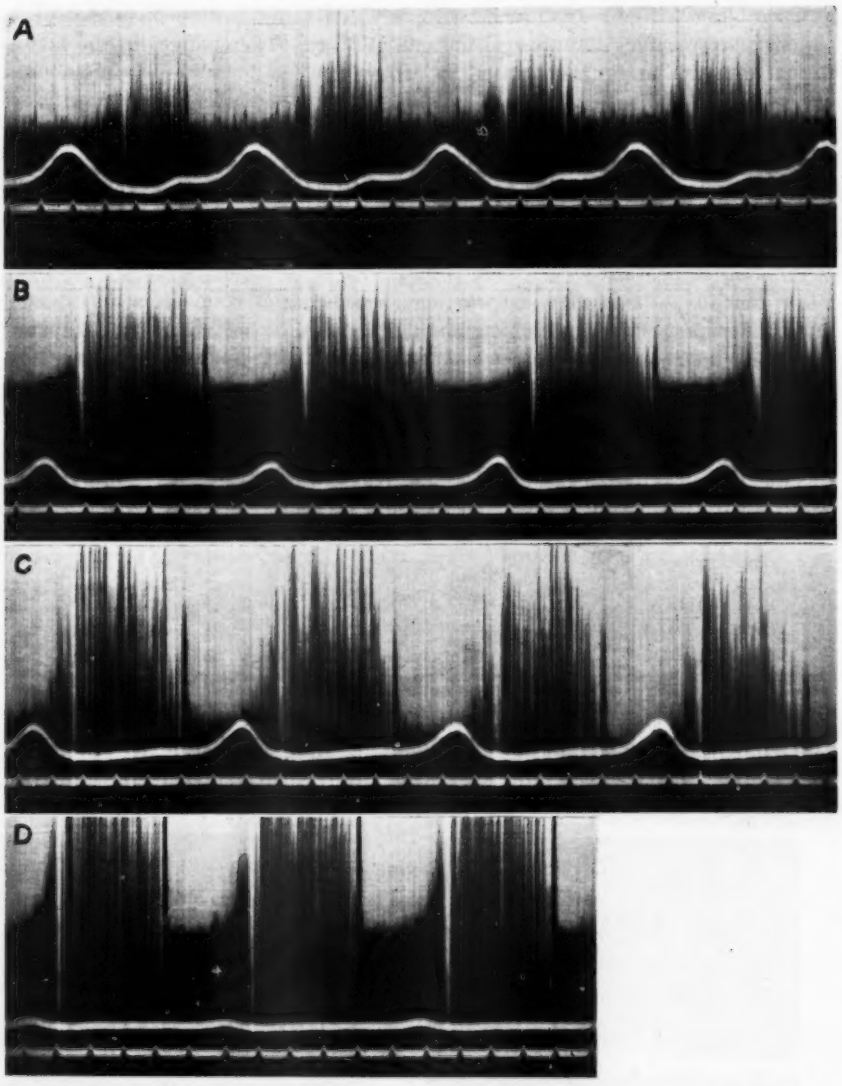

Figure 2. Oscillograph record from the descending fourth phrenic root, and tracheal record of the respirations in a rabbit weighing 2·14 kg. under dial and ether anaesthesia. The upstroke of the respiratory record corresponds to inspiration. (a), (b), and (d) are at $\frac{1}{20}$ and (c) at $\frac{1}{50}$ amplification. (a) is immediately before the injection of 1·0 mg. of mussel poison by a marginal ear vein, (b) 1 minute, (c) 2 minutes, and (d) 4 minutes after the injection, immediately before final failure of respiration. Time in one-fifth of a second.

dic stimulation of the phrenic nerves, there was a functional block in the transmission of respiratory impulses to the musculature. The changes in the action currents are similar to those caused by asphyxia. The bursts of impulses, each corresponding to the initial phase of inspiration, are of longer duration with increase both in the frequency and in the apparent amplitude of their component waves, but instead of the respiratory movements increasing in extent as in asphyxia they progressively decrease until respiration fails. These changes invariably follow the injection of a lethal dose of mussel poison in rabbits anaesthetized with dial. Failure of the centres is manifested by sudden cessation of the action currents in the nerve.

It remains to be ascertained whether the slowing of respiration and the asphyxia-like changes in the action currents in the phrenic nerve may not be caused by the fall of blood pressure which almost immediately follows the intravenous injection of mussel poison.

If a synchronous mechanical record of respiration and blood pressure be

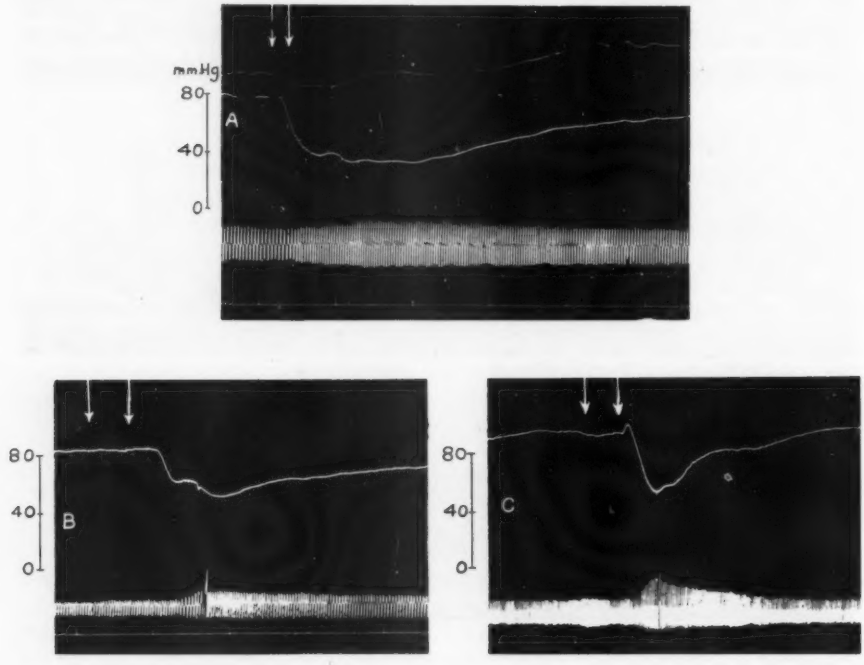

Figure 3. Blood pressure and tracheal respiratory records from rabbits weighing 2·95, 2·7, and 2·2 kg. under dial and ether anaesthesia. Between the arrows in (a) 0·025 mg. of acetyl choline, in (b) 0·5 c.c. Padutin (Kallikrein), and in (c) 0·5 c.c. Lacarnol were injected. In (a) there is also a plethysmogram of the right lower limb. Time in half minutes.

studied in anaesthetized animals in which a transitory fall of blood pressure is caused by the injection of vasodilator substances, there is invariably an increase in the amplitude of the respiratory movements when the mean blood pressure is at its lowest level. This occurs in the intact as well as in the completely "unbuffered" animal. In figure 3 are records of experiments in rabbits anaesthetized with dial and ether, showing the effect of the intravenous injection of acetyl choline 0·025 mg., "Padutin" Bayer 0·5 c.c., and "Lacarnol" Bayer 0·5 c.c. In each case there is a fall of the mean blood pressure of 40 mm. Hg. or more, which is accompanied by a transitory increase in the amplitude of the respiratory movement. The injection of acetyl choline causes a significant change in the rate of respiration. The fall of blood pressure caused by haemorrhage in the "unbuffered" animal in figure 1a is accompanied by an increase in the amplitude of respiration which quickens in the first phase and is only slowed significantly when the mean blood pressure is less than 10 mm. Hg.

The descending action currents in the fourth phrenic root of rabbits anaesthetized with dial, and in which acetyl choline, Padutin, and Lacarnol were injected intravenously, showed the same asphyxia-like changes which follow the injection of mussel poison.

An intact rabbit weighing 2·06 kg., anaesthetized with dial, received 0·075 mg. of acetyl choline intravenously; 40 seconds later the respiratory rate was slowed, and the bursts of impulses descending in the phrenic nerve were greatly increased in apparent amplitude and frequency of the component waves. These changes persisted for about a minute. 1·0 c.c. of Lacarnol injected about one hour later caused similar changes in the action currents, but no appreciable change in the rate of respiration.

Another rabbit weighing 1·93 kg., similarly anaesthetized, received 0·9 c.c. of Padutin. This was followed by an enormous increase in the apparent amplitude and frequency of the descending action currents in the phrenic nerve, which persisted for 2½ minutes. At the same time the respiration rate was increased.

It may be noted that the effects produced by acetyl choline are not analogous to those caused by Lacarnol, a preparation which resembles in its action the adenylic acid of Drury and Szent-Györgyi (1929), or Padutin—the Kallikrein of Frey and Kraut (1930). The vasodilator action of Lacarnol and Padutin resembles that of mussel poison, since full atropinization which completely abolishes the fall of blood pressure caused by acetyl choline is without effect upon the changes produced by the injection of Lacarnol, Padutin, or mussel poison.

In numerous experiments the intravenous injection of vaso-dilator substances has invariably produced changes in the action currents in the phrenic nerve resembling those caused by asphyxia, but the changes in respiratory rate were variable and relatively insignificant, and slowing of the rate of respiration similar to that regularly produced by moderate doses of mussel poison was never observed. The slowing of the respiration rate following the injection of mussel poison even in sublethal doses is therefore due to direct depressant action on the respiratory centres. The asphyxia-like changes in the action currents in the

phrenic nerve are caused in part by the fall of blood pressure due to the poison and in part by the asphyxia due to insufficient pulmonary ventilation. Since they occur in the "unbuffered" as well as in the intact animal, the effect of the fall in blood pressure must in part at least be due to deficient oxygenation and accumulation of products of metabolism at the centres.

We may now sum up the effects of mussel poison upon respiration. With large doses the central depressant action of the poison suffices to cause instant failure of respiration. With doses of the order of a single lethal dose the effect is more complicated. The depressant central action causes slowing of respiration. The associated fall of blood pressure causes stimulation of the centre, partly directly and partly through the carotid sinuses. The asphyxia-like response of the centre fails to be effective in increasing the respiratory movements since there is a block in transmission at the motor endings which may or may not be demonstrable by faradic stimulation. Asphyxia increases the disability of the centres and finally central failure occurs.

THE ACTION OF THE POISON ON THE VASO-MOTOR SYSTEM.

A number of experiments have been made upon cats and rabbits under dial anaesthesia, with ether for the necessary operative procedures, in order to analyse these effects. Plethysmograms of the lower limbs, spleen, and intestine have been recorded simultaneously with the carotid blood pressure. The general results obtained are illustrated in figures 4, 5, and 6. Figure 4 is the record obtained from a rabbit with the hind limb enclosed in an oncometer, and shows the response to a second intravenous injection of about 0·4 mg. per kg. of mussel poison. In this animal, as Leake and his colleagues have pointed out, there is a

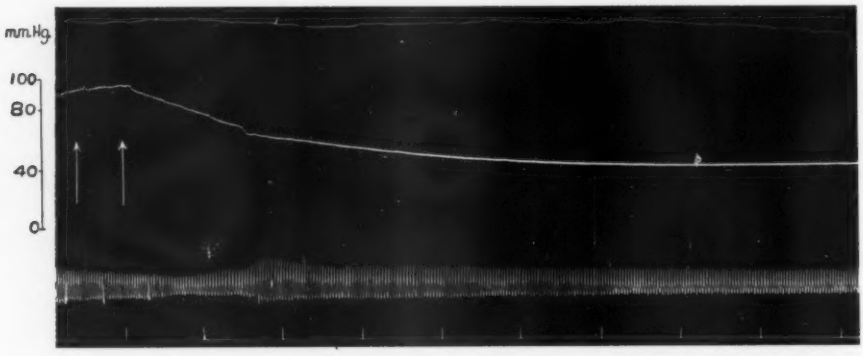

Figure 4. Blood pressure tracing, limb plethysmogram, and tracheal respiratory record from a rabbit weighing 3·08 kg. under dial and ether anaesthesia, showing the response to a second dose of 1·2 mg. of mussel poison. A final dose of 1·1 mg. of poison 16½ minutes later caused failure of respiration and death within 2½ minutes. Time in half minutes.

short initial rise followed by a fall of blood pressure. The plethysmogram shows a barely appreciable increase in limb volume during the early part of the fall of blood pressure. In contrast to the effects produced by powerful vaso-dilator

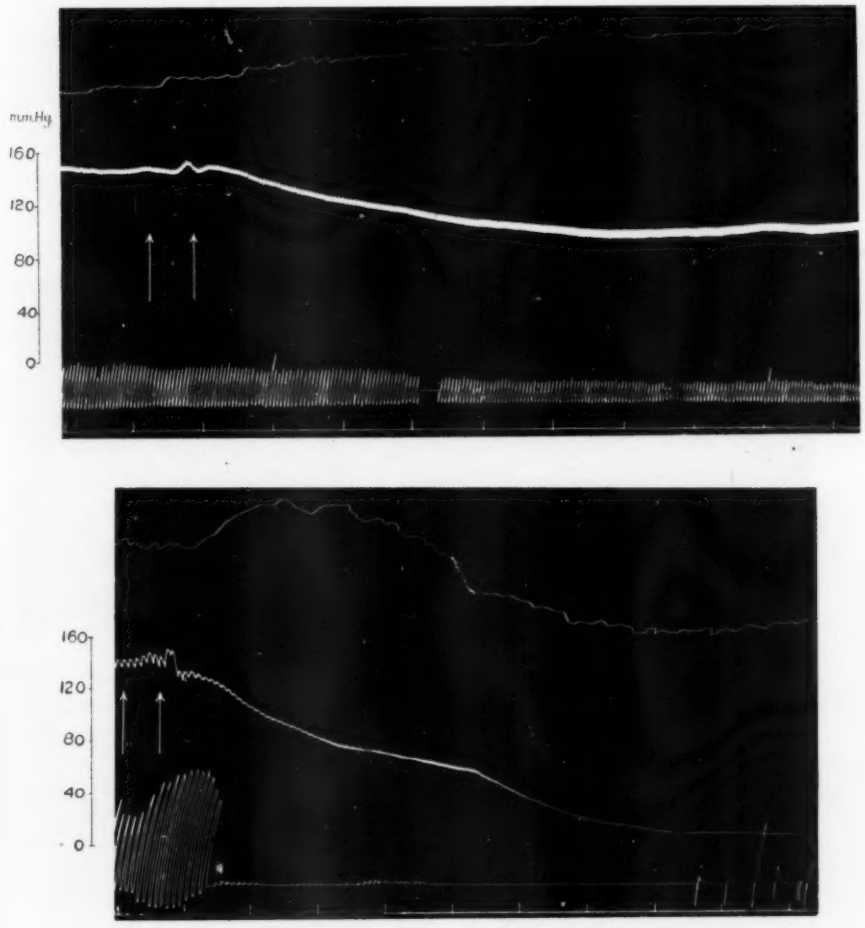

Figure 5. Blood pressure tracing, intestinal plethysmogram, and tracheal respiratory record from a cat weighing 4·1 kg., under dial and ether anaesthesia. Both vagi and one carotid sinus were intact. The upper panel shows the response to the injection of 2·0 mg. of mussel poison. The blood pressure recovered to 110 mm. Hg. in 6 minutes without recourse to artificial ventilation. The dose was repeated 38 minutes and 1½ hours later. The response to the last injection, which caused death in 5½ minutes, is shown in the lower panel. Time in half minutes.

drugs the volume changes in the limbs, though active, were always very small both in rabbits and cats, even though sensitive recorders were used.

Figure 5 shows the response to two intravenous injections of 2·0 mg. of mussel poison in a cat weighing 4·1 kg. The first injection caused a moderate fall of blood pressure accompanied by an increase in the volume of an intestinal loop. This injection was repeated 38 minutes and 1½ hours later. The last injection caused death in 5½ minutes. The plethysmogram showed a striking rise in intestinal volume corresponding to the initial fall of blood pressure and then a passive fall as the blood pressure fell still lower. Changes in the volume of the bowel due to muscular contraction of the walls of the enclosed loop frequently complicate intestinal plethysmograms. Since the smooth muscle is not directly affected by mussel poison the observed muscular activity is probably due to asphyxia.

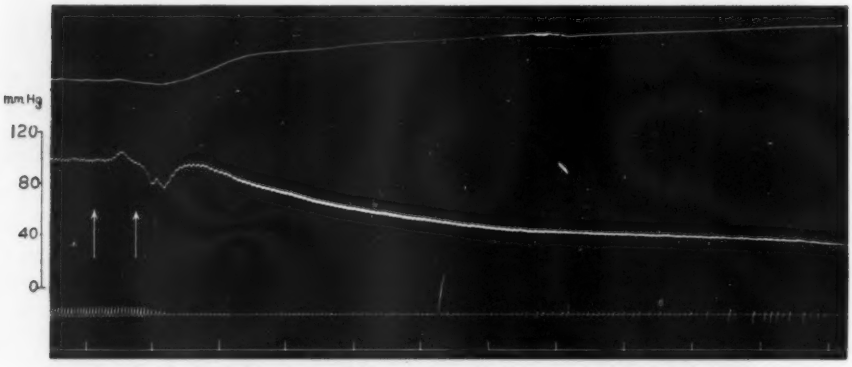

Figure 6. Blood pressure tracing, splenic plethysmogram, and tracheal respiratory record from a cat weighing 3·25 kg., under dial and ether anaesthesia. A first dose of 2·0 mg. of mussel poison caused the blood pressure to fall from 126 to 54 mm. Hg. The respirations did not fail. Forty-four minutes later, a second dose of 2·0 mg. of poison injected intravenously caused the changes recorded here. Respiration failed 5½ minutes after this injection. The animal was kept alive at a very low level of blood pressure (20 mm. Hg.) for nearly an hour under artificial respiration. Time in half minutes.

Figure 6 illustrates the typical blood pressure changes in a cat weighing 3·25 kg. which received two intravenous injections of 2·0 mg. of mussel poison at an interval of 44 minutes. The record shows the result of the second injection which caused failure of respiration in 5½ minutes. The blood pressure shows the typical initial fall, a secondary rise, and then a prolonged fall. This injection would have been fatal, but the animal was kept alive under artificial respiration. The spleen shows no significant change of volume with the initial rise and fall of pressure, though with a more delicate recorder active changes, that is, increase in splenic volume accompanying the fall and shrinkage with the secondary rise, were

observed several times. This record shows a considerable increase in the splenic volume accompanying the prolonged final fall of blood pressure.

The fall of blood pressure which results when mussel poison is injected intravenously in animals is due to peripheral vasodilatation, and affects principally the splanchnic region. The vessels in the spleen and intestines remain sensitive to second, third, or even fourth injections of mussel poison. However, animals which have been eviscerated or in which the splanchnic nerves have been divided, still show an appreciable fall of blood pressure following the injection of moderate doses of the poison. Figure 7 exemplifies this. It records the responses in a cat of 2·7 kg., in which all the splanchnic nerves had been divided, to successive doses of 2, 2, and 4 mg. of mussel poison. An interval of one hour separated the first and second, and one of 19 minutes the second and third injections. The first injection resulted in a moderate fall of blood pressure. The second gave a similar primary fall but only a very slight secondary fall of blood pressure. Following the third and largest injection the primary fall was evident, but the secondary fall was hardly perceptible.

The vascular response of the animal is independent of the "buffering" of the circulation provided by the carotid sinus and aortic reflexes. No essential difference in the vascular response to mussel poison has been detected between

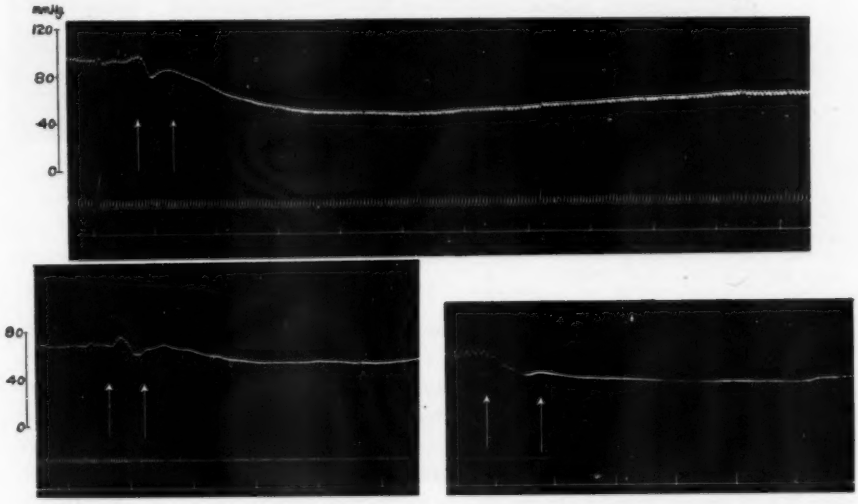

Figure 7. Blood pressure tracing, tracheal respiratory record, and plethysmogram of lower limb of a cat weighing 2·7 kg., under dial and ether anaesthesia, in which all the splanchnic nerves had been divided in the abdomen. The upper panel shows the response to the intravenous injection of 2·0 mg. of mussel poison; the left lower panel that to 2·0 mg. one hour later, and the right lower panel that to 4 mg. of mussel poison 19 minutes later. The respiration failed three minutes after the second injection. Time in half minutes.

the intact animal and that in which the carotid sinuses have been excluded and the vagi divided.

It might be argued that the anaesthetic necessary for these experiments plays an important part in the vascular reaction. This is not the case, since decerebrate preparations behave in a wholly similar manner.

Finally, it is of interest to observe the effect of mussel poison upon the spinal or decapitate preparation. Figure 8 is an example of such an experiment in a decapitate cat. The primary fall of blood pressure is present following three successive injections, though it is notably less in the second and third responses.

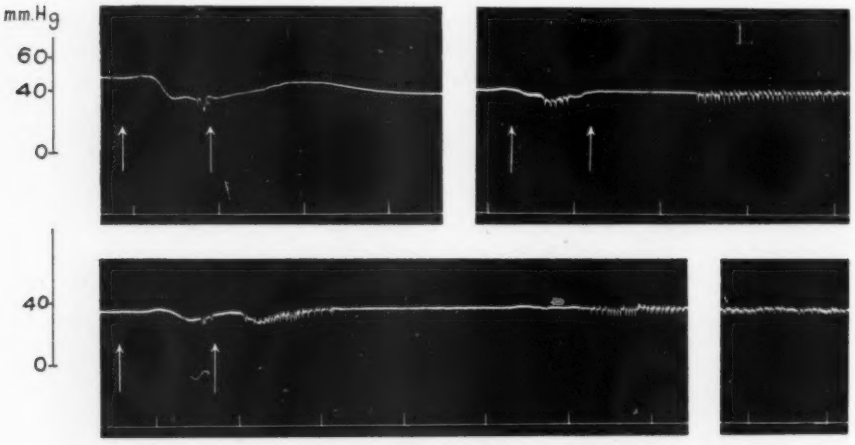

Figure 8. Blood pressure tracing from the carotid artery of a decapitate cat weighing 4·43 kg., showing responses to 10 mg. of mussel poison (the top left panel), 5 mg. given four minutes later (top right panel), 11·0 mg. 30 minutes after the first injection (lower left panel), though after the first injection (lower right panel) the blood pressure was well maintained. Following the second and third injections there was transient irregularity of the heart beat. Time in half minutes.

There is no appreciable secondary fall of blood pressure after any of these large doses. Indeed after the first injection there is a moderately prolonged rise of pressure. It is true that the blood pressure level is rather low throughout, and that the extent of response to vasodilator substances depends to a considerable extent upon the level of blood pressure, but in the intact animal smaller doses of mussel poison always cause a further fall of pressure if injected when the pressure is at a low level before recovery has taken place from a previous injection of poison. These experiments and similar observations on animals in which the brain was pithed after the spinal cord had been divided high in the cervical region, indicate that the action of the poison on the vascular system is almost wholly central.

Spinal and decapitate animals are the best preparations for the electro-cardiographic study of the action of the poison upon the heart. Large doses set up transitory periods of disordered heart action, and the blood pressure tracings are very suggestive of heart block. I have not, however, made adequate experiments upon this point, large doses of poison being required. In one or two experiments electro-cardiograms were taken, but the records were obtained when the effect was passing off and heart block was not demonstrated, though there was gross irregularity and prolongation of the P.R. interval.

PERIPHERAL EFFECTS OF THE POISON ON THE NERVOUS SYSTEM.

1. *The Curarizing Action of Mussel Poison in Frogs.*

This was studied *in vitro* using sciatic gastrocnemius preparations which were set up in pairs in wax-lined petri dishes separated into two compartments by a wax partition in which two slits packed with vaseline permitted the transit of the nerves. One muscle and one nerve were immersed in physiological saline solution (NaCl 0·65%, KCl 0·014%, $CaCl_2$ 0·024%, and $NaHCO_3$ 0·02%), and the corresponding nerve and muscle in saline containing mussel poison. In Table 2 are set out the results of a group of experiments carried out at 15°C. The frogs used were *Hyla aurea* freshly captured in November. The largest distance separating the secondary from the primary coil at which contraction of the poisoned muscle occurred is expressed as the numerator, the figure obtained at the same time for the muscle in saline being the denominator. If no contraction occurred with the coils in contact the result is written as 0.

TABLE 2.

The Curarizing Action of Mussel Poison on Gastrocnemius Sciatic Preparations of HYLA AUREA.

Concentration.		Effect on irritability of muscle. By indirect stimulation.	By direct stimulation.
1: 200,000	1½ hours	0/13	21·5/26·5
	4½ hours	0/0	8·5/24
1: 500,000	3¼ hours	0/14·5	19/28
	5 hours	0/6·5	11·5/27
1: 1,000,000	5½ hours	0/7	23/28
1: 2,000,000	6½ hours	27·5/11	12/25
Saline controls (mean)	5½ hours	49	27·5

It will be observed that mussel poison has a very powerful curarizing action on frog nerve endings, as has already been shown to be the case for mammalian endings in the intact animal. In addition it has a direct poisoning action upon muscle and nerve. The motor nerve endings are most susceptible, next the nerve fibres, and finally the muscle itself. In a concentration of 1 : 200,000 of the dry poison, curarization occurred in 1½ hours, and the conduction of nerve immersed in poison was destroyed in 4½ hours. Complete loss of irritability of muscle did not occur within the time during which the preparations were observed.

2. *The Paralysant Action of Mussel Poison on Sensory Nerve Endings.*

A striking feature of poisoning in man is the occurrence of paraesthesias—"pins and needles", numbness, and itching. It seemed possible that these might be due to peripheral action upon sensory nerve endings. The poison was tested upon the dorsal skin preparation used by Bronk (1929) and the peroneal toe muscle preparation described by Matthews (1931).

In testing tactile endings, four to six dorsal cutaneous nerves were prepared and tested in succession after the endings connected with the nerve first tested were paralysed. The nerves were placed one at a time in a moist chamber, across cotton thread electrodes of the type used by Adrian. The excised dorsal skin was placed external surface uppermost on the thin cork roof of a vulcanite moist chamber, and the nerve entered the chamber through a slit. The nerves awaiting test were kept moist between the skin and the cork. Tactile stimuli were applied by light touch with the tip of a primary wing feather of a fowl. The skin was kept wet throughout the experiment with poison solution of the strength under test.

In the study of muscle spindles, the apparatus used was that devised by Matthews (1931), which was made for me by my colleague, Mr. E. Hughes. No attempt was made to secure single ending preparations, since it was advantageous to use preparations in which many end organs were functioning. Under these conditions each experiment tests the most resistant of a group of organs of varying susceptibility. The tension on the tendons was applied by a 5 gm. weight at the end of a lever whose motion was controlled by a plunger in a dashpot containing heavy oil. The physiological saline solution, or the mussel poison dissolved in this solution, in the small vulcanite trough which contained the preparation, was changed every five or ten minutes throughout the experiment. The stocks of saline solution and dilute poison were kept at constant temperature in thermos flasks. In the period between observations the nerves were kept immersed in the solution in the trough. The frogs used were *Hyla aurea*. They were freshly caught in November, and used within a few days of capture.

The toxin was found to have an extremely rapid paralysant action upon the tactile endings of the skin of this frog. In tests carried out at 15°C. concentrations of 1 in 2,000,000; 1 in 1,000,000, and 1 in 500,000 of the dry preparation caused complete paralysis of nerve endings in 60, 12, and 2 minutes. Since the dorsal nerves of the frog are very fine, it seemed possible that part of this effect might be upon the nerves themselves. Experiments were therefore carried out in which a portion of a nerve under test was bathed with poison in a concentration of 1 in 1,000,000 and 1 in 500,000 at 15°C. This was found to be without effect upon the action currents for periods of one hour and half an hour respec-

tively. Mussel poison was now applied to the skin, and the usual rapid paralysis of tactile nerve endings occurred.

Tests of the action of the poison upon muscle spindles were carried out at several different temperatures, and gave the results set out in Table 3. The frogs were used shortly after capture during the latter half of November.

TABLE 3.

Paralysant Action of Mussel Poison on Muscle Spindles.

Concentration of toxin.	Temperature.	Time to failure of action current.
1: 1,000,000	8°C.	2½ hours.
1: 500,000	8°C.	58 minutes.
	8°C.	1¼ hours.
	4°C.	5 hours.
1: 200,000	13°C.	2 hours 43 minutes.
1: 100,000	10°C.	29 minutes.
1: 50,000	3°C.	1 hour 5 minutes.

The poison has a powerful paralysant action upon these endings, being effective in concentrations up to 1 in 1,000,000 of the dry preparation. To demonstrate that there is a genuine effect upon the nerve endings experiments like the following were performed.

Two peroneal toe muscle preparations from a single frog gave good action currents in the nerves audible with the loud speaker at $\frac{1}{50}$ of the total amplification when tension was applied with a 5 gm. weight to the tendons. They were set up as in the experiments for testing curarizing action, one nerve and one muscle being immersed in physiological saline solution, and the corresponding muscle and nerve in 1: 200,000 mussel poison in physiological saline solution. The experiment was carried out at room temperature, 15·5°C. The solutions were changed at 10 minute intervals from stocks kept at 8°C. in thermos flasks. After one hour both preparations were tested, being put up in physiological saline solution in the testing bath. The nerve endings of the preparation, the muscle of which had been in poison solution and the nerve in saline, were now found to be paralysed, and no action currents could be produced in the nerve by tension on the tendons. The preparation, the nerve of which had been immersed in poison solution and the muscle in saline, gave unaltered responses. This was now put up in 1: 200,000 solution of mussel poison, and ceased to give any response to tension on the tendons after half an hour.

From this group of experiments it is clear that mussel poison has a powerful effect on the amphibian peripheral nervous system. The tactile endings in skin are most sensitive to the paralysant action of the poison, the motor endings and muscle spindles are also very susceptible, and nerve fibres themselves are blocked by relatively high dilutions of the poison. The poison is much more potent in these dilutions than any of the snake venoms. I have not been able to show any reversal of these effects in *in vitro* preparations.

In the early moments after the application of the poison in high dilution to the skin, and also to the toe muscle preparations, there was regularly some en-

hancement of the response to stimuli as judged by the amplified loud speaker record, but at no time was there evidence of spontaneous firing of the endings brought about by the poison. It remains, therefore, uncertain whether the paraesthesias observed clinically are of central or peripheral origin, and if the latter, whether they may not be caused by the vasomotor action of the poison.

SUMMARY.

The mussel poison prepared by Sommer is a neurotoxin with both central and peripheral actions. The central effects are predominantly upon the cardiovascular and respiratory centres. The peripheral effects are predominantly upon the nerve endings, both motor and sensory.

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THE MECHANISM OF THE ACTION OF ETHYLENE ON CELL PROCESSES

by

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INTRODUCTION.

That ethylene could affect cell metabolism has been known from the beginning of the century (Neljubow, 1911), and its industrial application has become a commercial actuality for the ripening and colouring of fruit.

Very little progress, however, has been made in the attempt to explain this accelerating action of ethylene. Whereas Chace and Church (1927) said that the "ripening" was only a colour change in the skin, Harvey (1928) held that ethylene actually brought about biochemical changes in the cells such as hydrolysis of starch, protein cleavage, and the disappearance of amines and organic acids; and they made the suggestion that the gas acted as a "coenzyme" for the hydrolytic ferments. In his studies on seeds germinated in ethylene, Harvey (1915) showed that all those changes which take place naturally in a growing seed with the effluxion of time, such as an increase in the soluble substances, sugars, amino acids, etc., at the expense of the insoluble ones (protein, starch, etc.), are hastened by the presence of ethylene.

That ethylene can bring about cell proliferation is definite, because epinastic growth and hastened abscission of leaves can be brought about by its presence. In breaking the dormancy of potatoes with ethylene chlorhydrin, Denny (1926) observed that this compound did not affect the tissue extract itself but stimulated the tissue to produce peroxidase and amylase in greater quantity. He also demonstrated that cell metabolism was affected since ethylene (1 part in 100,000) more than doubled the rate of respiration of lemons.

With regard to the effect of ethylene on cells and enzymes in vitro the results have been a little confusing, although the majority have favoured the view that ethylene did not act on the enzyme itself but on the cell. Regeimbal and Harvey (1927), employing crushed pineapple, found that the proteolytic activity was enhanced when ethylene was added, and also that chemical analysis of treated and

untreated pineapples revealed an increase of reducing sugars in the treated fruit. From this they concluded that ethylene increased both the action of invertase and the proteolytic enzymes. It should be pointed out, however, that they were dealing with cells and not with cell-free enzymes.

Englis and Zannis (1930) were the first to employ enzymes prepared free from cell material. The enzymes tested were diastase and invertase, and no difference in the rate of hydrolysis could be detected between the treated material and the control. Hirschfelder and Ceder (1930) could not obtain any increased activity by the action of ethylene on pepsin, trypsin, or lipase; whilst Englis (1931) obtained similar negative results using emulsin and salicin. Englis concluded that "the results of these experiments suggest that a very complete system of reactions is taking place. The question might be raised as to whether or not studies as to the effect of ethylene with pure substrate material should be abandoned. Perhaps mixtures of enzymes may be studied and some key reaction in the chain found. If not, one must come to the conclusion of Denny that the chemicals seem to produce larger amounts of enzymes and their effect is not a direct one upon the enzymes".

On the other hand the work of Nord (1928, 1930), which was undertaken mainly to elucidate the mechanism of enzyme action, is not in accordance with the above conclusion. He has stated that an increased fermentation was obtained with cell-free zymase, and also that ethylene acted on the cell as an anaesthetic, giving three stages of narcosis (see Discussion).

In the experiments recorded in this paper it was found that ethylene does not affect the activity of isolated enzymes, but that it acts on the intact living cell.

EXPERIMENTAL RESULTS.

Yeast cells provided an excellent medium for the experimental work, as they are cells of a single definite type, rich in two important enzymes, invertase and zymase.

The effect of ethylene on the inverting action of yeast was investigated on both intact and dead cells.

The Effect of Ethylene on Living Yeast.

Ethylene was passed for 2 minutes into 100 ml. of a 20 p.c. sucrose solution, placed in a constant temperature bath at 39°C.; after 10 minutes, 100 ml. of a suspension of washed yeast cells (*S. cerevisiae*) were added. At definite intervals 20 ml. portions were removed and added to 3 ml. of $\frac{M}{10}$ Na_2CO_3, centrifuged, and any change in rotation measured in a polarimeter. A control experiment was carried out without the addition of ethylene. After 3 hours a further addition of 100 ml. of sucrose solution was made.

There was a definite increase in the rate of inversion of the sucrose (see Table 1).

TABLE 1.

Time.	Rotation. Control.	Ethylene treated.	Time.	Rotation. Control.	Ethylene treated.
0 mins.	14·60	14·60	0 mins.	14·15	14·15
80 „	6·65	6·60	60 „	8·10	8·00
140 „	2·75	2·65	125 „	3·75	3·60
	Second addition of sugar at 215 mins.				
270 „	7·50	7·30	240 mins.	10·00	9·80
335 „	4·90	4·55	330 „	6·50	6·25
410 „	2·00	1·60	390 „	4·40	4·10
430 „	1·00	·60	450 „	2·70	2·35

The Effect of Ethylene on Dead Yeast.

The previous experiment was repeated, except that the temperature of the bath was maintained at 52°C., at which temperature yeast is killed. (Table 2.)

TABLE 2.

Time.	Rotation. Control.	Ethylene treated.	Rotation. Control.	Ethylene treated.
0 mins.	13·30	13·30	13·00	13·00
25 „	8·60	8·60	10·10	10·10
50 „	5·80	5·80	6·55	6·60
110 „	3·50	3·50	3·70	3·70
180 „	2·50	2·55	1·75	1·80

According to Bokorney (1915), 0·05 p.c. sulphuric acid kills yeast (i.e. budding will not take place again).

In Table 3 are shown the results obtained after the yeast had been killed by 0·5 p.c. sulphuric acid, and then carefully washed to remove all acid.

TABLE 3.

Time.	Rotation. Control.	Ethylene treated.	Rotation. Control.	Ethylene treated.
0 mins.	13·20	13·20	13·10	13·10
25 „	10·40	10·40	10·30	10·30
50 „	7·80	7·80	7·60	7·65
110 „	4·00	4·00	3·50	3·50
200 „	1·80	1·80	1·40	1·45

The Effect of Ethylene on Alcoholic Fermentation by the Living Cell.

The view is held by Nord (1930) that ethylene acts on cell-free zymase, and further, when acting on yeast during fermentation, the cell undergoes three phases of narcosis. Because these conclusions would not allow the acceptance of the idea that ethylene acts only on the cell and not on the enzyme, it was thought desirable to repeat the work.

20 ml. of a 20 p.c. glucose solution, saturated with CO_2, were taken and ethylene passed through for 1 minute. The mixture was placed in a constant temperature bath for 10 minutes, and then 2 gm. of pressed top yeast were added. The CO_2 evolved was measured in an apparatus similar to that described by Harden and Young, and modified by Paine (1911). Readings were taken every 5 minutes.

As shown in Graph 1, there was an increased fermentation rate, but no evidence was obtained of a narcotic action.

The above experiment was repeated with yeast killed by 0·1 p.c. sulphuric acid. In agreement with the results obtained with invertase, no increase in the rate was obtained.

THE MECHANISM OF ETHYLENE ACTION.

Having established that ethylene affects the cell but not the enzyme, it was desirable to determine how this effect is brought about.

Harvey (1928) made the suggestion that the gas increased the permeability of the cell, thereby allowing the substrate and enzyme to mix more freely. O'Sullivan (1890) stated that invertase did not come out of fresh live yeast into the surrounding medium; but if chloroform were added it altered the permeability, and the invertase appeared in the surrounding liquid. If ethylene increased the permeability it might have a similar effect.

Yeast was allowed to stand in contact with ethylene water overnight, centrifuged, and the invertase content of the supernatant liquid tested. No invertase came out of the cell, from which it may be concluded that ethylene does not alter the permeability in regard to this enzyme.

Paine (1911) has determined the permeability of the yeast cell to various electrolytes and, employing his method, an attempt was made to see if ethylene influenced the permeability.

20 gm. of washed and pressed yeast were added to 50 ml. of NaCl, through which ethylene had been bubbled for 2 minutes, and the mixture was placed in the ice chest at 4°C. for 20 hours, centrifuged, and the NaCl in 20 ml. was estimated. From this could be calculated the amount which had penetrated into the cell. The experiment was repeated using Na_2HPO_4 in place of NaCl. The results are given in Table 4.

TABLE 4.

	Gm. of NaCl taken up by 50 gm. of yeast.	
	1.	2.
Control	0·18 gm.	0·19 gm.
Ethylene treated	0·18 gm.	0·19 gm.
	Gm. of P_2O_5 taken up by 20 gm. of yeast.	
	1.	2.
Control	0·45 gm.	0·55 gm.
Ethylene treated	0·45 gm.	0·55 gm.

It is evident that ethylene has not altered the permeability of the cell to these electrolytes.

The Electrical Conductivity of a Yeast Suspension in Relation to Ethylene Treatment.

If a system consist of cells suspended in an electrolyte, the conductivity of the suspension is less than that of the electrolyte because the cells hinder the flow

of the ions, and if the permeability of the cells should increase this would allow freer movement of the ions and so lead to an increased conductivity.

Yeast was washed twice in saline (of the same concentration that it was to be suspended in later) and pressed. Saline solutions were made up of the following concentrations, 0·25 p.c., 0·5 p.c., and 1 p.c. Before suspending the yeast in the saline, ethylene was passed through for 4 minutes. 2 gm. of yeast were suspended in 10 ml. of saline of a definite concentration, and after standing 2 hours the conductivity was measured at 25°C. The suspension was then centrifuged, and the conductivity of the menstruum measured. The results are shown in Table 5.

TABLE 5.

		Conductivity (× 10).				
		Yeast.		Menstruum.		
Conc. of Saline.	Time.	Cont.	Ethyl.	Cont.	Ethyl.	Saline.
·25%	2 hrs.	·03547	·03406	·04087	·04023	·04170
	2 „	·03549	·03376	·04070	·04087	
	18 „	·03477	·03346	·04087	·04087	
	18 „	·03549	·0337	·04023	·04001	
·50%	2 „	0·6485	·06381	·05401	·07401	·07491
	2 „	·06620	·06510	·07491	·07495	
	18 „	·05485	·06255	·07341	·07341	
	18 „	·06510	·06255	·07491	·07495	
1·00%	2 „	·1131	·1104	·1306	·1261	·1345
	2 „	·1131	·1109	·1251	·1251	
	18 „	·1122	·1086	·1261	·1251	
	18 „	·1131	·1086	·1306	·1306	
* L solution	2 „	·1187	·1084	·1442	·1430	·1536
	2 „	·1233	·1109	·1412	·1412	
	18 „	·1187	·1084	·1442	·1430	
	18 „	·1187	·1109	·1442	·1424	

* Because the cell wall may be permeable to salts other than NaCl, a solution was made up containing: NaCl, ·8 gm.; $CaCl_2$, ·03 gm.; $MgCl_2$, ·01 gm.; K_2HPO_4, ·1 gm.; H_2O, 100 gm. (designated L. solution).

From these results it may be concluded that ethylene caused a decrease in the conductivity of a yeast suspension, but did not affect the conductivity of the fluid in which the cells were suspended, i.e. there was no evidence of increased protoplasmic permeability.

The Effect of the Addition of Phosphate on Fermentation in the Presence of Ethylene.

It is well-known that toluene and similar substances bring about some disorganization of the yeast cell so that it shows the typical reaction to added phosphate, i.e. increased rate of fermentation and formation of hexosephosphates.

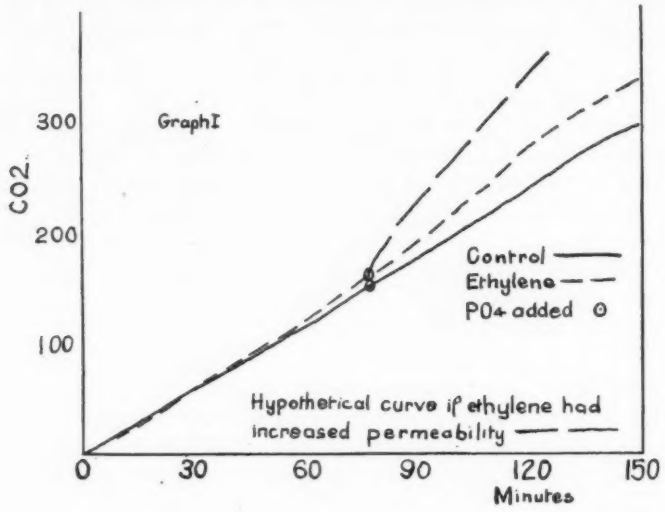
This effect could not be obtained by the action of ethylene on yeast (see graph 1).

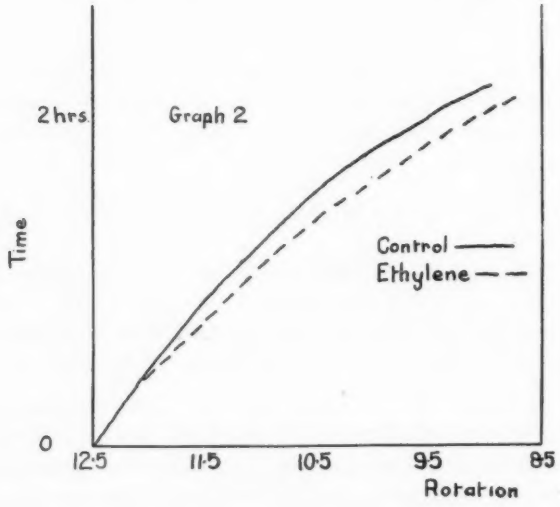
The Effect of Ethylene on the Invertase Content per Unit Weight of Yeast.

Although it has been shown that ethylene does not bring about its increased action by alteration of cell permeability, it may be attributable to an increase in

the production of enzyme by the cell. The method employed to investigate this aspect was a modification of that described by Miller (1921).

20 ml. of a 2 p.c. suspension of top yeast were added to 250 ml. of Williams' culture media, the culture media having had ethylene passed through for 4 minutes. It was then incubated for 24 hours at 31°C., filtered, 0·4 gm. of the yeast taken, and 25 ml. of water and 0·5 ml. of toluene added, and the whole shaken for 4 hours at room temperature. The mixture was then





added to 100 ml. of a 10 p.c. sucrose solution, and placed in the bath at 50°C. At the end of 1 and 2 hours the rotation was measured.

An increased amount of invertase in the treated yeast was demonstrated. See Graph 2.

DISCUSSION.

From a consideration of the experimental part it will be seen that ethylene does not in any way affect a cell-free enzyme or any enzyme action brought about by the dead cell, while there is an accumulation of evidence that ethylene can bring about an increased activity within a living cell. This conclusion agrees with the tentative one put forward by Englis.

On the other hand, according to Nord, ethylene hastens the action of cell-free zymase by preventing the destructive action of proteolytic ferments present in the preparation, by becoming adsorbed on to the enzyme. Several criticisms can be raised against this statement. The first is that even his own results are not consistent in showing an increase, for while some curves show an increase others show an actual decrease of the same order; and secondly, if yeast be killed with sulphuric acid it does not show such an increase, although the proteolytic enzymes could still exert their so-called destructive effect. Moreover, this is the only example in the literature of a cell-free enzyme being activated by ethylene, and as such may be regarded with suspicion.

Nord has also postulated that ethylene acts on a cell as an anaesthetic and brings about three stages of narcosis.

a. A phase of external narcosis with lessened activity;

b. A phase of increased permeability with heightened action;

c. A phase of internal narcosis with permanent inhibiting effect.

These concepts are contrary to all the established facts of ethylene action.

Nord tried the effect of ethylene on the carboxylase of living yeast, but obtained no evidence of these three phases. A study of graph 1 in the present work reveals no such stages, and the accelerating action which commences early is maintained until the end. The second stage is supposedly due to increased permeability, but all the evidence (Tables 4 and 5) points to no alteration in the cell wall. Finally, the effect of ethylene on fruit, etc., is definitely beneficial, while Nord infers that, especially in the last stage, ethylene would be toxic. It is thus evident that the conclusions of Nord cannot be brought to bear heavily against the theory that ethylene acts on the cell itself.

With regard to the mechanism of this cell-stimulating action of ethylene, it may be said that it is not one of increased permeability, but that ethylene in some manner alters the cell metabolism so as to bring about an increased production of enzymes. Experiments on the conductivity of the yeast suspension in the presence of ethylene showed a slight decrease in the conductivity; this could be due either to a decrease in the permeability or to an enlargement of the cell. So if

ethylene stimulates the production of enzymes, may not the cell enlarge to bring about this production?

The exact mechanism for this additional production of enzyme is difficult to explain, mainly because of the lack of knowledge[1] in regard to the elaboration of ferments by the cell.

SUMMARY.

It has been shown that the rates of fermentation and inversion by living yeast are increased in the presence of ethylene, but these increases were not obtained if the yeast were dead, nor with cell-free preparations of the enzymes.

Ethylene does not alter the permeability of the cell wall.

Evidence is presented to show that ethylene can increase the enzyme content of yeast per unit weight. It must therefore bring about its stimulating action by causing an increased production of enzyme by the cell.

ACKNOWLEDGMENT.

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[1] Hohler (1929) has said that during the action of maltase in living yeast on maltose, the actual amount of enzyme increases. It may be possible that ethylene produces a similar stimulation to the production of enzymes.

HAEMOLYSIS BY AUSTRALIAN SNAKE VENOMS

4. FURTHER STUDIES ON THE HAEMOLYSIN OF THE COPPERHEAD.

by

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(Submitted for publication 27th March, 1935.)

In paper No. 3 of this series (Holden, 1934) a description was given of the influence of hydrogen ion concentration on the haemolysis of washed rabbit erythrocytes by the venom of the Australian Copperhead snake (*Denisonia Superba*), together with an account of the inhibition of this haemolysis by certain proteins. This paper contains a description of the effects on this reaction of several other substances, a method of measuring the velocity curve of haemolytic reactions, and an account of the effects of various additions to the form of such curves.

It is not easy to find any satisfactory method of expressing the results of inhibitions and accelerations of these haemolyses in general quantitative terms. Although, as noted before, some form of logarithmic expression, suggesting the removal of part of the venom through adsorption, fits the curve of an inhibition experiment moderately well—at all events over a section of the time-concentration curve covering as great a range of concentrations as Ponder (1933, 1) employed to establish the formula—the correlation is not very exact over the whole range of concentrations (about ten times that of Ponder) employed. Again the fact that other considerations militate against the acceptance of such an adsorption scheme is also against the use of a formula implying this idea.

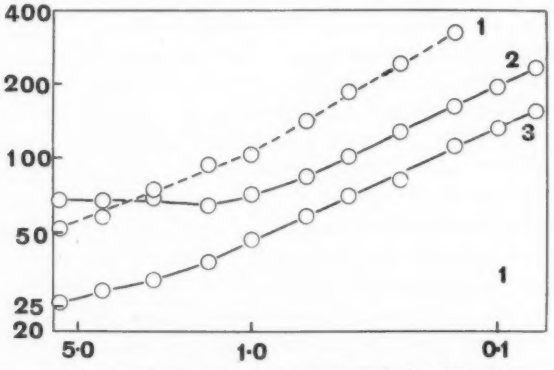
An examination of the time-concentration curves at pH 6·6 or 7·7 shows that the flattened portion of the curve occurs between the same concentrations of venom in the case of the control series as in that of the series containing the inhibitory substance. Again, the auto-inhibition shown at high concentrations of venom occurs in the presence of an inhibitory protein over the same range of venom concentrations as in the control at all hydrogen ion concentrations. That is, the relative forms of the curves are such as to indicate, not a removal of a portion of the venom, but the action of the same amount of venom, in the presence of the inhibitor as in the control, upon erythrocytes rendered more resistant. The auto-inhibition and the flattened portion of the curve at pH 6·6-7·8 suggest the presence, in the venom itself, of an inhibitor whose affinity, either for the haemolysin or for the cells, is affected by its concentration and by that of hydrogen

ions. It is possible to construct an hypothesis that accelerations and inhibitions are due to variations in the affinity between the haemolysin and this inhibitor in the venom. It would not, however, on this basis, be easy to include accelerations in very dilute solutions of venom. On the whole, the most satisfactory theory would at present appear to be that variations in the velocity of haemolysis are due more to changes in the envelope of the erythrocyte than to changes in the amount of venom available.

The Effect on Haemolysis of Substances contained in Erythrocytes.

In the previous paper it was erroneously stated that the acceleration of haemolysis observed in the presence of laked rabbit corpuscles was due to the liberated haemoglobin. It was subsequently found that if the cells were laked at pH 6·0 and centrifuged the solution of haemoglobin thus obtained was a feeble inhibitor, while the accelerator was contained in the lipoids accompanying the stroma protein. Kyes (1910) had found this to be true for guinea-pig cells, using cobra haemolysin. The stroma protein of rabbit cells is an inhibitor. If, however, it is used without first extracting the lipoids the accelerative effect of the latter far outweighs its own effect.

Small stocks of the lipoids of rabbit, sheep, and horse erythrocytes were prepared as follows. The cells were washed four times with saline, and then extracted twice with ten volumes of absolute alcohol. The filtered extract was evaporated to dryness *in vacuo*. The residue was extracted with absolute alcohol and filtered. The filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in dry ether, filtered, and the lipoids precipitated from the filtrate by the addition of two volumes of dry acetone. The precipitate was washed with acetone and dried in a vacuum desiccator. When required for use it was suspended in a suitably buffered



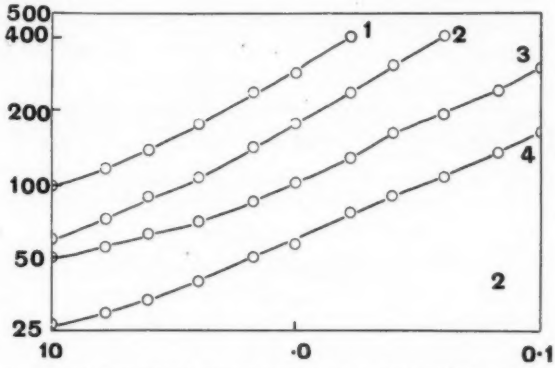
Abscissae: mg. of copperhead venom per c.c. of washed rabbit cells.
Ordinates: time of haemolysis in minutes. Both are plotted logarithmically.

Fig. 1. The effect of $CaCl_2$ at pH 6·0.

Curve 1. Control.
2. 0·2 g. $CaCl_2$ per c.c. of cells.
3. 0·002 g. $CaCl_2$ per c.c. of cells.

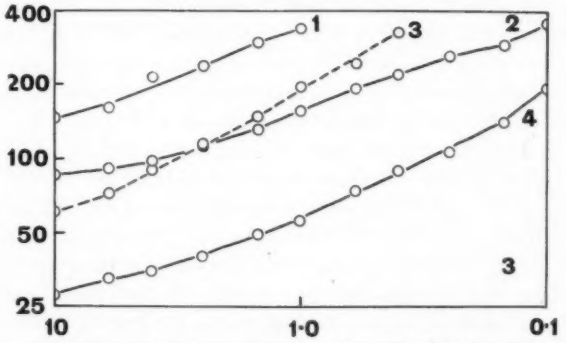
saline. The technique of determining time-concentration curves was described in the previous paper.

The extract of rabbit cells was a powerful accelerator, more so in acid solution (Fig. 6). That of horse cells was a fair accelerator, while that of sheep cells inhibited the haemolysis of washed rabbit cells, being most potent in alkaline solution (Figs. 3, 5).



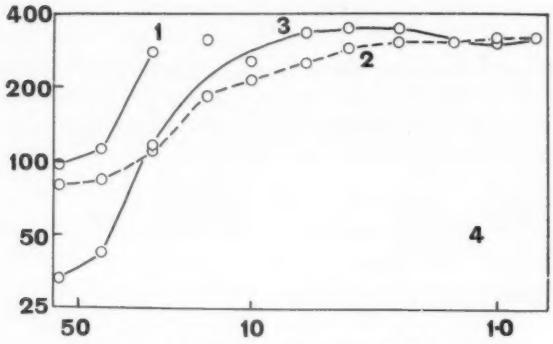
Abscissae: mg. of copperhead venom per c.c. of washed rabbit cells.
Ordinates: time of haemolysis in minutes. Both are plotted logarithmically.

Fig. 2. Inhibition at pH 6·0 by ox serum albumin of the acceleration by $CaCl_2$.
Curve 1. 0·01 g. albumin per c.c. of cells.
2. Control.
3. 0·002 g. $CaCl_2$ and 0·01 g. albumin per c.c. of cells.
4. 0·002 g. $CaCl_2$ per c.c. of cells.



Abscissae: mg. of copperhead venom per c.c. of washed rabbit cells.
Ordinates: time of haemolysis in minutes. Both are plotted logarithmically.

Fig. 3. Inhibition at pH 6·0 by sheep cell extract of the acceleration by $CaCl_2$.
Curve 1. Extract of 1·6 c.c. sheep cells per c.c. of rabbit cells.
2. Extract as 1, and $CaCl_2$ as 4.
3. Control.
4. 0·002 g. $CaCl_2$ per c.c. of rabbit cells.



Abscissae: mg. of copperhead venom per c.c. of washed rabbit cells.
Ordinates: time of haemolysis in minutes. Both are plotted logarithmically.

Fig 4. Effect of $CaCl_2$ at pH 7·8.

Curve 1. 0·02 g. $CaCl_2$ per c.c. of cells.
2. Control.
3. 0·004 g. $CaCl_2$ per c.c. of cells.

Note. 0·1 g. $CaCl_2$ per c.c. of cells: no haemolysis.

The Effect of Calcium Chloride.

The effects on haemolysis due to the presence of calcium chloride appear to be somewhat complex, depending on the concentration not only of this salt but on those of hydrogen ions and of venom. At pH 6·0 a concentration of 0·003 p.c. of the anhydrous salt showed a powerful acceleration which became more marked as the concentration of venom decreased (Fig. 1). Increasing the amount of calcium chloride did not much alter the situation until a concentration of 0·3 p.c. was reached, when it became slightly inhibitory with the highest concentrations of venom and decidedly less of an accelerator with the low concentrations of venom. At pH 7·7-7·8 its behaviour is peculiar (Fig. 4). In concentrations of 0·008 p.c. it accelerated high concentrations of venom and had an inhibitory effect on low concentrations. In concentrations of 0·03 p.c. it inhibited complete haemolysis except for the strongest venom solutions employed, where it greatly delayed haemolysis. On the other hand, in the presence of small amounts of venom only, 0·03 p.c. or 0·06 p.c. calcium chloride brought about a rapid haemolysis of a portion of the cells, and the process did not reach completion within the period of observation. The 0·06 p.c. calcium chloride inhibited completely the haemolysis by the strongest venom solutions employed. The subject will be further discussed in the section dealing with the velocity of haemolysis. At this stage it may be observed that these peculiarities serve to emphasize the complex nature of such haemolyses.

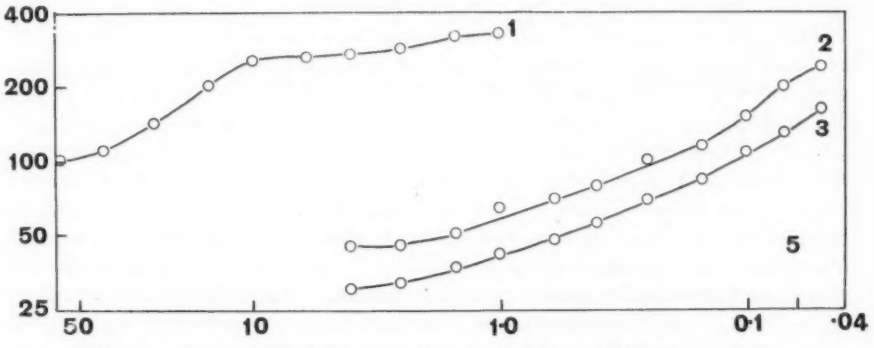
Two other substances may be mentioned in passing. Methyl alcohol in a

concentration of 14 p.c. did not haemolyse the rabbit cells but acted as a powerful accelerator to haemolysis by the venom. Cholesterol only delayed the haemolysis very slightly. This may be due to the fact that its insolubility compelled the use of suspensions to avoid possible damage to the cells from organic solvents.

A series of experiments in which an inhibitor and an accelerator were used in combination indicated that the substances mentioned above exert their influences almost independently of each other's presence. The resultant curve represents fairly well the sum of the curves for the separate substances. This applies to such pairs as cholesterol—methyl alcohol, calcium chloride—ox serum albumin, and calcium chloride—sheep cell extract. An increase in the concentration of hydrogen ions acts in favour of the accelerative effects and against the inhibitory effects, even when, as for example with calcium chloride, they are due to one substance (Figs. 2, 3).

Lecithin.

Kyes (1910) showed that, in the presence of lecithin, cobra venom haemolysed sheep cells, which are ordinarily unattacked by this venom. He found that a powerful lysin was formed as a result of the interaction of the venom and the lecithin which could haemolyse the cells in the absence of the former. In order to study the behaviour of a lecithin in connection with copperhead venom a specimen of hen ovo-lecithin was made by the method of Levene and Rolfe (1927). It was precipitated twice as the cadmium chloride compound, and was practically



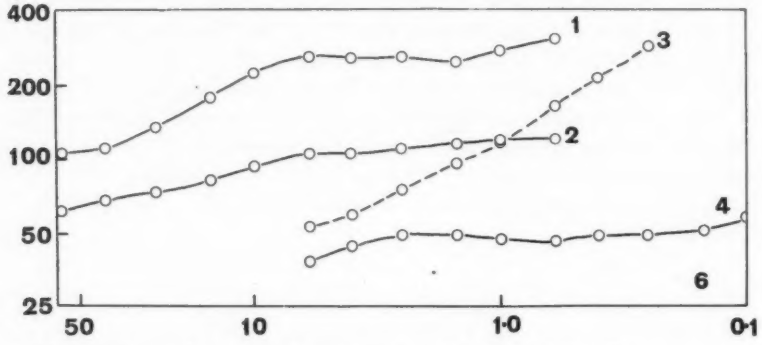
Abscissae: mg. of copperhead venom per c.c. of washed rabbit cells.
Ordinates: time of haemolysis in minutes. Both are plotted logarithmically.

Fig. 5. Inhibition at pH 7·7 by sheep cell extract of the accelation by lecithin.

Curve 1. Control.
2. Extract of 3·3 c.c. of sheep cells and 0·005 g. lecithin per c.c. of rabbit cells.
3. 0·005 g. lecithin per c.c. of rabbit cells.

Note. The sheep cell extract alone completely inhibited haemolysis.

free from cholesterol. By the action of copperhead venom upon this lecithin was formed a powerful haemolysin which haemolysed not only washed rabbit cells but also those of the sheep and ox. Lecithin, previously treated with copperhead



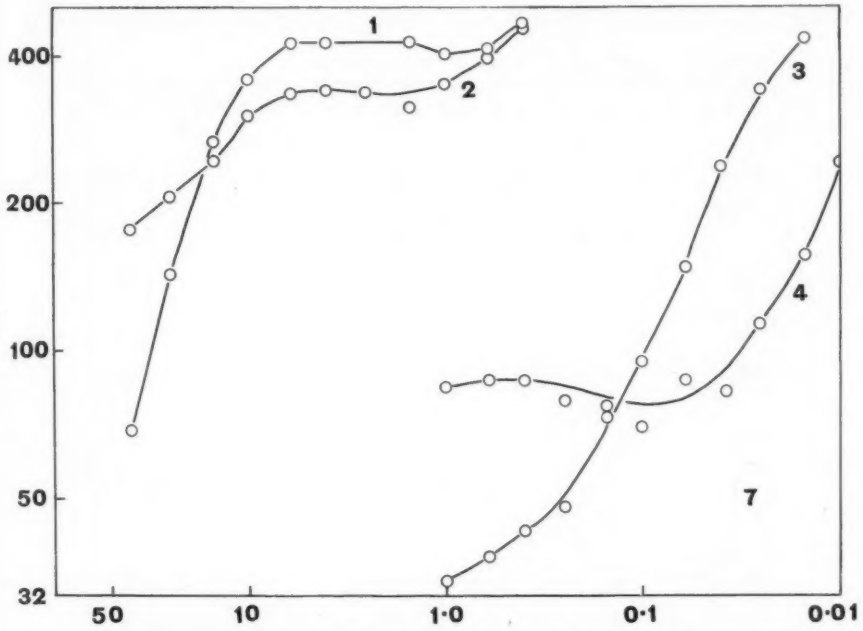
Abscissae: mg. of copperhead venom per c.c. of washed rabbit cells.
Ordinates: time of haemolysis in minutes. Both are plotted logarithmically.

Fig. 6. The acceleration at pH 6·0 and pH 7·7 by rabbit cell extract.

Curve 1. pH 7·7 control.
2. pH 7·7 extract of 3·2 c.c. of cells per c.c. of cells haemolysed.
3. pH 6·0 control.
4. pH 6·0 extract as Curve 2.

venom at room temperature for a couple of hours, haemolysed washed rabbit cells at 37°C. almost instantly. This lysin formation occurred more rapidly at pH 7·7 than at pH 6·0. So much was this the case that an amount of lecithin that produced only a feeble acceleration at pH 6·0 produced an intense one at pH 7·7, multiplying the apparent activity of the venom over two hundred fold, and actually haemolysing the cells faster than the same quantities of venom and lecithin at pH 6·0. Cholesterol inhibited the reaction to some extent if a large amount were dissolved with the lecithin in alcohol, the mixture evaporated to dryness and the solid mixture suspended in water. The haemolysis, however, was completed in the presence of the mixture in about one-fifth of the time that would be required in the absence of both of the constituents; the inhibitory power of the cholesterol decreased with decrease in the concentration of venom. Ox serum albumin and the lipoid extract of sheep cells inhibited the haemolysis of washed rabbit cells in the presence of lecithin to a small extent compared with their inhibition in its absence (Figs. 5, 7). It would appear that where haemolysis by copperhead venom is observed *in vivo* it is dependent on the lecithin present in the serum. The low concentration of venom present in the blood, the reaction, and the presence of the proteins, seem to rule out any idea of an unaided

haemolysis of the cells by the venom. It would appear that the slowest reaction in the process of haemolysis of washed cells, which determines the apparent velocity, is not the action of the venom on the lecithin of the envelope but something



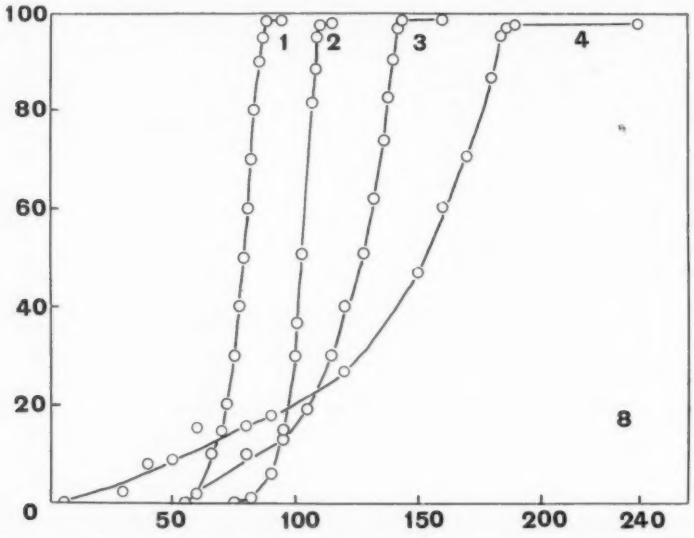
Abscissae: mg. of copperhead venom per c.c. of washed rabbit cells.
Ordinates: time of haemolysis in minutes. Both are plotted logarithmically.

Fig. 7. Combined effect of $CaCl_2$ and lecithin at pH 7·7.

Curve 1. 0·004 g. $CaCl_2$ per c.c. of cells.
2. Control.
3. 0·005 g. lecithin per c.c. of cells.
4. 0·004 g. $CaCl_2$ and 0·005 g. lecithin per c.c. of cells.

antecedent to that. The idea of a prior proteolysis is tempting; it would agree with the inhibitory powers of such proteins as are adsorbed to the surface of the erythrocyte. Hen ovalbumin stands, as it not infrequently does, aloof. The effect of sheep cell extract is less clear. Sheep red cells contain nearly as much "lecithin" as do rabbit cells, and little more cholesterol, which is not apparently very important. The quantitative differences are certainly insufficient to explain the differences in stability towards venom. It may be noted, in this connection, that copperhead venom rapidly dissolves the stroma of laked rabbit cells, yielding a clear red solution, while it is without apparent effect on rabbit stroma that has

been centrifuged from the dissolved haemoglobin or on the stroma of laked sheep cells. The final act in the course of haemolysis appears to be the solution of the stroma protein. Unfortunately, little is known of the constitution of the phosphorus-containing lipoids of erythrocytes, but it would seem that differences among various species exist. The acceleration by rabbit cell extract is greater at



Abscissae: time in minutes from the moment of mixing the cells with the venom.
Ordinates: percentage haemolysis.

Fig. 8. Velocity of haemolysis at pH 6·0.

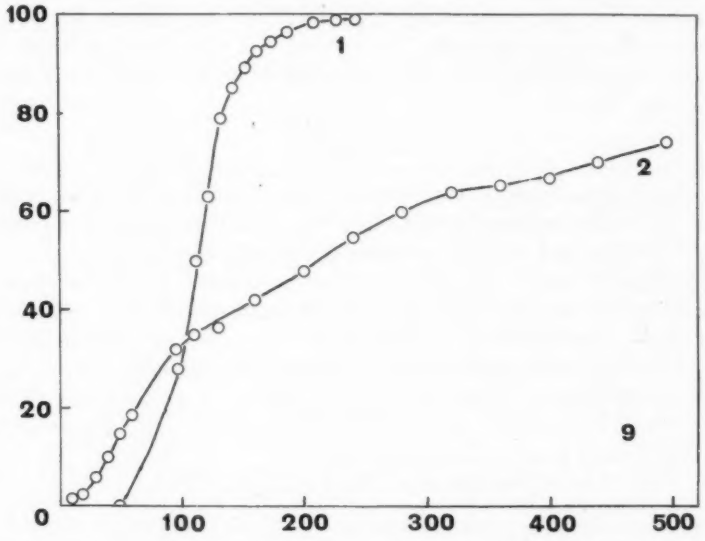
Curve 1. 5·0 mg. of copperhead venom per c.c. of cells.
2. 0·5 mg. of venom and extract of 0·5 c.c. of cells per c.c. of cells haemolysed.
3. 2·0 mg. of venom per c.c. of cells.
4. 0·2 mg. of venom and 0·007 g. $CaCl_2$ per c.c. of cells.

pH 6·0 than at pH 7·7. If the accelerator present be of the lecithin type there would appear to be also present a fairly powerful inhibitor. The diagram which shows the combined effect of lecithin and calcium chloride is of some interest (Fig. 7). In the presence of lecithin alone the flattened portion of the curve is entirely lost, while the addition of calcium chloride restores it but at lower values of both time and concentration of venom. Beyond suggesting that the flattened portion in the control curve may be due to a similar opposition between an accelerator and an inhibitor whose power varies with the concentration of the haemolysin, the theoretical approach to the problem of the mechanism of haemolysis is not much aided thereby.

The Determination of the Velocity of Haemolysis.

Ponder (1923) determined the velocity of the haemolysis of human red cells by saponin and other chemical agents by measuring at intervals the amount of light transmitted from a powerful lamp on to a radiometer through the cell suspension. For the experiments in this paper a simpler apparatus was employed.

A differential colourimeter was constructed by shifting both cups and plungers of a Bausch and Lomb instrument to one side. The second plunger was fixed to the lower side of the carrier of the top cup. The displaced plunger was replaced by a right angle prism, through which was



Abscissae: time in minutes from the moment of mixing the cells with the venom.
Ordinates: percentage haemolysis.

Fig. 9. Velocity of haemolysis in alkaline solution.

Curve 1. pH 8·0. 40 mg. of venom per c.c. of cells.
2. pH 7·7 1·67 mg. of venom and 0·1 g. $CaCl_2$ per c.c. of cells.

viewed the reaction chamber. This was 8 mm. deep, with parallel vertical glass sides. It was immersed in a water-bath maintained at a constant temperature. The illuminating system consisted of a 60 watt opal lamp and two mirrors. Ground glass screens were introduced in each light path. All stray light was carefully excluded by suitable screens. In the lower cup were placed laked cells, and in the upper a solution of a mixture of a commercial dye, "nigger brown", and a little methylene blue. This imitated surprisingly well the tint of a cell suspension under the conditions of illumination employed. The instrument was calibrated each morning on the cell suspension to be used, by placing in the reaction chamber known mixtures of laked and intact cells from the suspension to be used, and noting the reading on the colouri-

meter scale at which the two sides matched. With this apparatus a number of velocity curves were determined.

The essential shape of the curves was somewhat similar to that observed by Ponder for haemolysis by sodium taurocholate: a latent period followed by an assymetric S-shaped curve. The velocity was, however, nearly constant from about 20 p.c. to over 90 p.c. haemolysis. In alkaline solution it fell off a little earlier than at pH 6·0. When the total time of haemolysis was fixed, other factors, for example accelerators or inhibitors (Fig. 8), had apparently little effect on the shape of the curve, with the important exception of calcium chloride. At all concentrations of hydrogen ions the presence of calcium chloride greatly diminished the latent period. At pH 7·7 in higher concentrations the shape of the curve was found to be completely changed. The velocity fell steadily after 30 p.c. of the cells had been haemolysed. It is at present impossible to suggest any explanation of this result (Fig. 9).

SUMMARY.

The haemolysis by copperhead venom of washed rabbit cells is accelerated by the lipoids of rabbit cells and inhibited by those of sheep cells. It is accelerated by hen ovo-lecithin and by small amounts of calcium chloride.

A method for measuring velocity curves of haemolysis is described. The velocity curve for copperhead venom is of an assymetric S-shape preceded by a latent period. Small amounts of calcium chloride decrease greatly this latent period. Larger amounts at pH 7·7 alter the shape of the curve.

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THE DISTRIBUTION OF VITAMIN C IN THE ORGANS OF THE FOX *(VULPES VULPES)*.

by

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INTRODUCTION.

The cytological demonstration of vitamin C was first described by the present author in a series of papers during 1933. The only other authors to work on this subject were Giroud, Leblond, and collaborators during 1934.

The original work was carried out on the adrenal gland since it was the organ which was known to contain probably the highest concentration of the anti-scorbutic vitamin. Leblond and other workers have since published a certain amount of information on the form of vitamin C in the cells of certain of the other organs and tissues of the body. The present paper constitutes a fairly complete but brief survey of the form which the vitamin C takes in the cells of the various organs of the body.

MATERIAL AND TECHNIQUE.

The original preparations of the present author were made by impregnation of the tissue with silver nitrate following fixation in the vapour of formaldehyde.

This method was soon discontinued, owing to the fact that portion of the vitamin C in the tissue was destroyed by the fixative. It was found that an alcoholic solution of silver nitrate gave good results, the mixture performing simultaneously the dual function of impregnation and fixation. Some very beautiful preparations were obtained by this method, particularly of the human foetal adrenal. Another method used by the present author has been the cutting of frozen sections of the fresh tissue, and their subsequent immersion in acidified silver nitrate solution; these gave a very good result, particularly when lipoid preparations were made on the same sections.

Giroud and Leblond have used a rather different technique. They kill the animal, wash out the blood from the vessels with an isotonic solution of levulose, and then inject varying concentrations of silver nitrate acidified with acetic acid. This they allow to act for different periods of time, usually about twenty minutes, and they subsequently run photographic "hypo" through the vessels to "fix" any unreduced silver nitrate.

Leblond and his co-workers also tested numerous reducing agents, commonly found in the body, against the acidified silver nitrate solution, and found that they did not reduce it.

The substances tested were: Adrenalin, tannin, hydroquinone, various sugars (including levulose), glucuronic acid and various acids of a dual sugar-phosphoric acid nature, e.g. pentose phosphoric acid; also glutathione, cysteine, and creatinine.

The acidified silver nitrate test for vitamin C then is, as far as we know at present, specific, and may be safely regarded as an indication of the presence of vitamin C.

The animals used for this work were foxes (*Vulpes vulpes*), and a similar technique to that of Leblond's has been adopted, after the animals were killed by strangling or anaesthesia.

DISTRIBUTION OF VITAMIN C IN TISSUES HAVING A LOW CONCENTRATION.

Pancreas.

The acinar cells (Fig. 1) of the pancreas did not stain very black, and the cells under high power showed the presence of only a few granules. The granules were large and distributed evenly throughout the cells, there being no particular association between the granules and either the nucleus or the cell membrane. At times the aggregation of three or four granules around the nucleus could be seen, but this was not a constant feature.

The cells of the islets of Langerhans (Fig. 2) did not possess a greater amount of vitamin C than the acinar cells, and some of them appeared to possess less, only three or four granules being observed. The distribution of the granules within the cells appeared fortuitous.

Spleen.

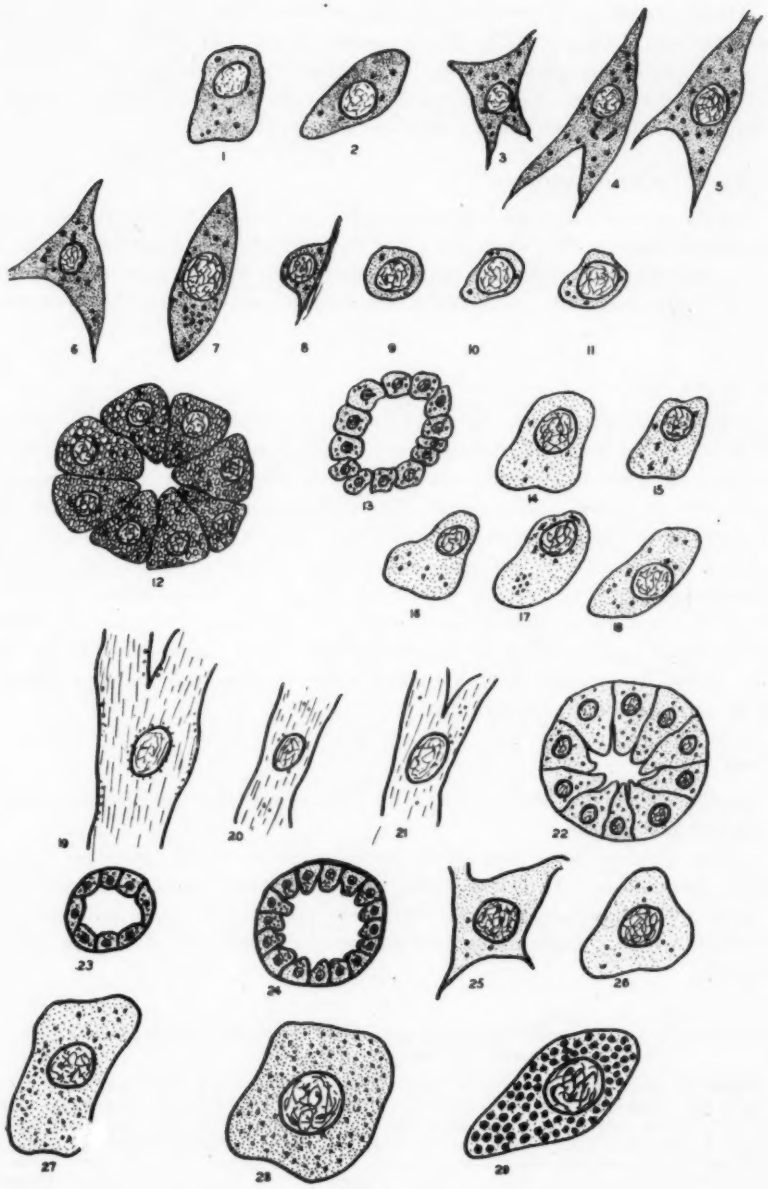
The spleen appeared to contain more vitamin C than a number of other organs—the granules appearing most frequently in the cells of the stroma.

These cells were of various shapes, and in most of them fifteen or twenty large granules could be observed (Figs. 3-7). In one cell nearly fifty granules could be seen, twenty of them being large and aggregated at one pole of the nucleus and merging gradually into the smaller granules which were scattered through the cytoplasm. In another cell three large granules could be seen on one side of the nucleus and one large one on the other side—scattered through the remainder of the cytoplasm were about fifteen small granules.

Most of the granules were of a rounded nature, some being rather irregular. In a few of the cells, however, quite definite rods were observed—some straight and a few curved, and of varying lengths.

Figures showing distribution of Vitamin C granules in the cells of various tissues. The large granules represent the vitamin.

Figure 1. Acinar cell of pancreas. **Figure 2.** Cell from islet of Langerhans: pancreas. **Figures 3, 4, 5, 6, and 7.** Cells from splenic pulp. **Figures 8, 9, 10, and 11.** Lymphocytes. **Figure 12.** Acinar cells of sub-maxillary gland. The granules of vitamin may be seen as three or four large black granules amongst the secretion droplets. **Figure 13.** Cells of a cardiac gland of the stomach. **Figures 14, 15, 16, 17, and 18.** Cells from cardiac region of the stomach. **Figures 19, 20, and 21.** Disposition of vitamin C in myocardial cells from ventricle of heart. **Figure 22.** Collecting tubule of kidney. **Figure 23.** Descending tube of Henle: kidney. **Figure 24.** Ascending tube of Henle: kidney. **Figures 25, 26, and 27.** Cells from the glomerulus: kidney. **Figure 28.** Liver cell. **Figure 29.** Endothelial cell from marrow capillary.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All the lymphocytes present in the spleen showed the presence of a few granules of vitamin C (Figs. 8-11). A few contained only two or three granules, but five or six was a more usual number. The granules were rounded and were scattered in the cytoplasm. None occurred in the nucleus. The reticular cells of the capsule seemed to contain quite a number of granules.

Sub-Maxillary Salivary Gland.

All the cells of the acini appeared packed with droplets of secretion, and between these droplets were scattered a few large granules of vitamin C (Fig. 12). They were rounded and did not appear to have any special association with the droplets. Most cells only contained a few granules, seven being the largest number observed.

Alimentary Tract.

Leblond has shown that the epithelial cells of the intestine of the rat have quite a number of rounded granules—he assumes that they are absorbed from the food. For the present work the cardiac region of the stomach has been examined. All the cells showed the presence of a few granules in the cytoplasm. The granules were, however, of uneven size, and some were even in the form of straight or curved rodlets. Ten or eleven was the usual number present. In the cells of the cardiac glands of this region also vitamin C granules could be observed (Figs. 13-15). There were approximately half a dozen to a cell. In one of the stomach cells there was a group of about one dozen granules of various sizes in the distal region of the cytoplasm; and surrounding the nucleus was an aggregation of slightly curved rodlets and granules.

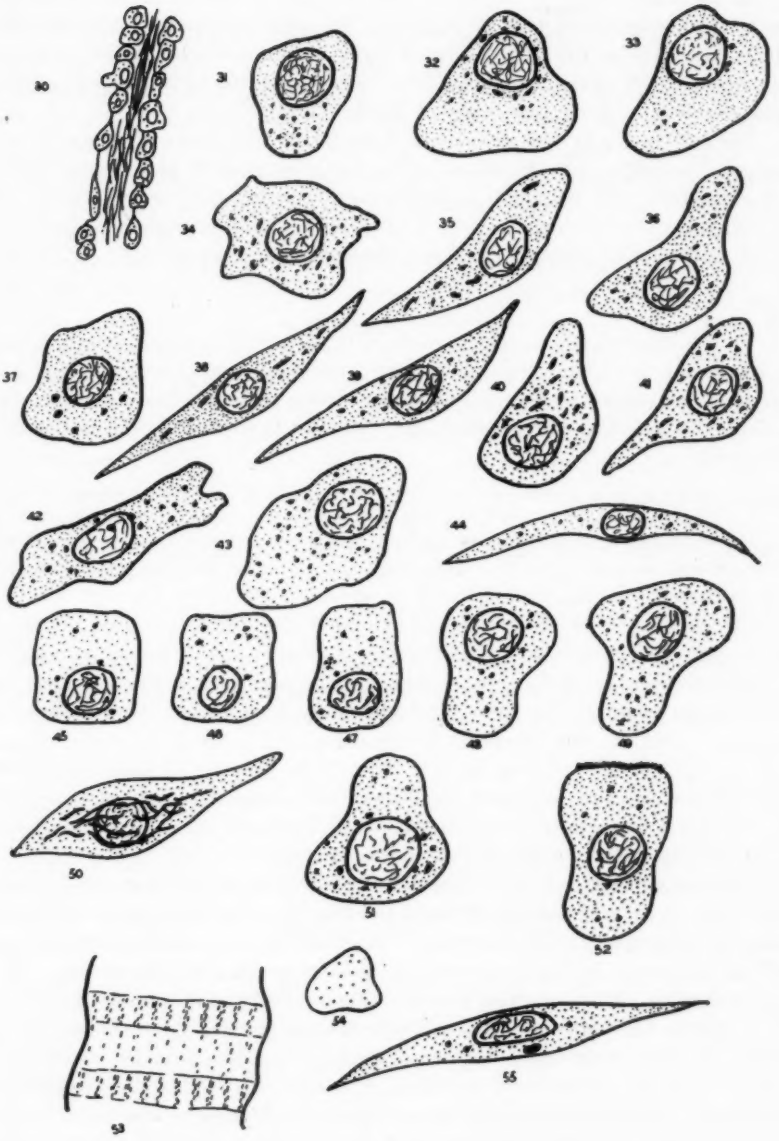
Kidney.

Leblond has found the vitamin C in this region to be aggregated chiefly in the glomerular regions.

All the kidney cells in the present preparations showed the presence of argentophilic granulation. The cells of the collecting tubules possessed both rodlets and granules disposed chiefly in the distal cytoplasm but not orientated in any particular direction; eight to ten granules or batonettes were present in each cell (Fig. 22).

Figures showing distribution of Vitamin C granules in the cells of various tissues. The large granules represent the vitamin.

Figure 30. Portion of alveolus of lung. **Figures 31–43.** Cells from lung alveoli. **Figure 44.** Endothelial cell from lung capillary. **Figures 45–49.** Cells from bronchioles of lung. **Figure 50.** A connective tissue cell from the trachea. **Figure 51.** Cartilage cell from the trachea. **Figure 52.** Ciliated epithelial cell from the trachea. **Figure 53.** H.P. Single muscle fibre to show rod shaped vitamin C granules aggregated chiefly in the dark lines, and orientated parallel with the muscle fibrillae. **Figure 54.** T.S. Single muscle fibre. **Figure 55.** Perimysial connective tissue cell.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In the descending tube of Henle (Fig. 23) approximately half a dozen granules were observed in each of the cells and similarly in the cells of the ascending tube of Henle (Fig. 24). In the latter the granules occupied almost exclusively the distal portion of the cytoplasm. No rodlets were found in the cells of either the descending or ascending tubes of Henle.

The cells in the various glomeruli (Figs. 25, 26, 27), and even the cells in the same glomerulus, varied very considerably in the number of argentophil granules they exhibited. The majority possessed about a dozen large granules, but some cells contained a very large number with a condensation in certain regions of the cell, in some near the nucleus and in others near the cell membrane.

Liver.

Numerous granules could be observed in the cytoplasm and attached to the nuclear membrane (Fig. 28). There were no intra-nuclear granules. Only large, rounded granules were observed, no rodlets being present. The Kupffer cells of the liver did not show any greater aggregation of the vitamin than the ordinary liver cells.

The Respiratory Tract.

In the trachea, the ciliated epithelial cells possessed a few (some half dozen) granules disposed irregularly throughout the cell and showing no relationship with the basal granules of the cilia (Fig. 52).

The cartilage cells of this region showed slightly more granules, which tended to aggregate round the nucleus (Fig. 51). Some of the connective tissue cells showed a series of long filaments directed in the long axis of the cell and partly enclosing the nucleus (Fig. 50). The cells of the bronchioles all contained a few vitamin C granules, some possessing only about half a dozen, and a few as many as twenty; they were all granular. In some cells a group of half a dozen very fine granules could be seen near the nucleus; the majority of the other granules were, however, twice this size. The endothelial cells of the lung capillaries also showed the presence of vitamin C granules (Fig. 44).

Leblond and his co-workers postulated that vitamin C is associated with the respiration of the cell and therefore in the alveoli of the lung, where respiratory exchanges are rapid and continuous; it was thought that the alveolar cells might contain large quantities of the vitamin. This was not found to be the case. There were a number of vitamin C granules in each cell, but the number varied very considerably; in some cells only six or seven occurred, in others as many as twenty or thirty. The granules were round or angular, large or small, and in some cells batonettes occurred as well as granules. In some cells, too, there was a tendency towards perinuclear condensation; in others there was none. In some cells a few, very large, granules occurred. The disposition of the vitamin in these cells is shown in Figs. 30-43 and 45-49.

Muscular System.

The muscles investigated were the ventricle of the heart, and a portion of the brachialis.

Vitamin C granules occurred only in small numbers in the myo-cardial muscle. In some fibres the granules were aggregated around the nucleus and on the membrane of the muscle cell, with no granules in the intervening region of the cytoplasm. In other cells the granules were fewer in number and were scattered indiscriminately throughout the cytoplasm of the cell—there was no perinuclear condensation (Figs. 19-21).

Ordinary voluntary muscle showed the presence of rodlets of vitamin C in the muscle fibres. These rodlets were aggregated chiefly in the region of the dark line of the muscle fibre, and in addition appeared to be situated between the muscle fibrils, being orientated with their long axes parallel with the fibrils. It was difficult to be quite certain whether the granules of vitamin were inter or intra fibrillar. There were only relatively few of these rodlets present in the muscle fibre, but they were all disposed in the manner stated. The connective tissue cells between some of the muscle fibres showed also the presence of argentophile granules (Figs. 53 and 54). In one cell (Fig. 55) there was quite a large mass situated in the distal region of the cell near the nucleus, and three very much finer granules in the cytoplasm extending on either side of the nucleus (the cell being flattened).

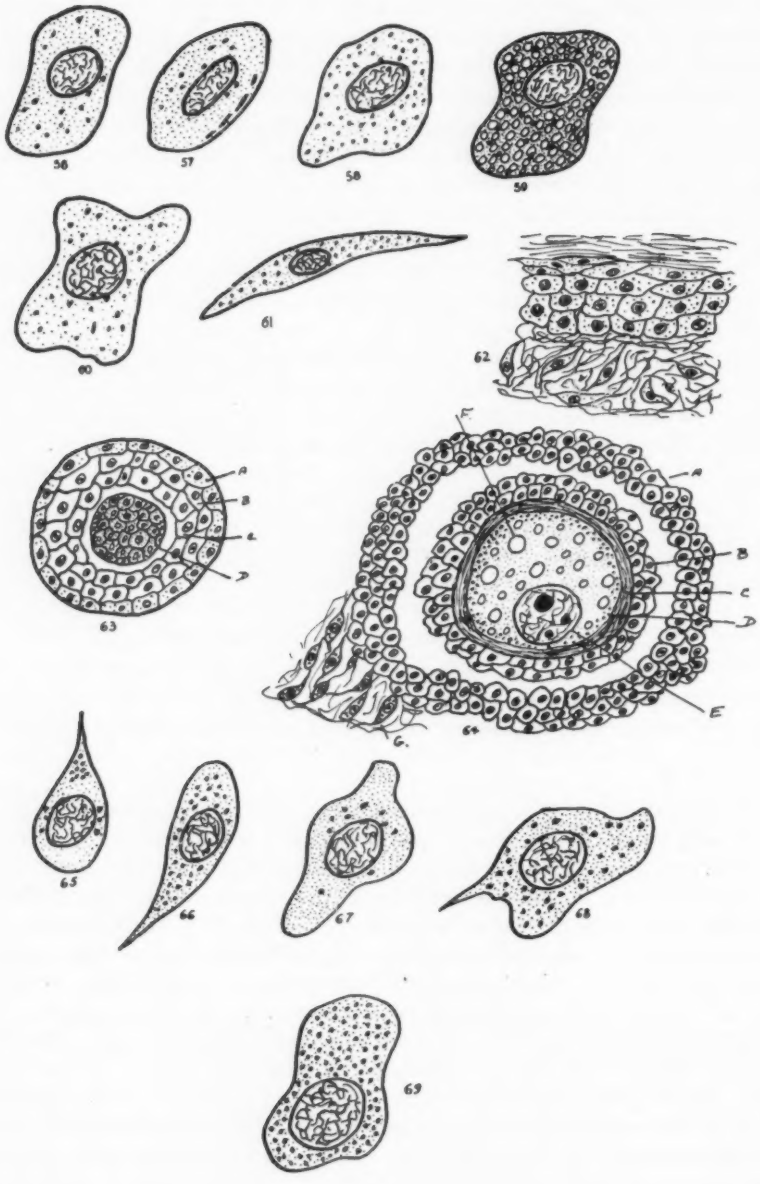
Marrow.

The endothelial cells of the marrow were found to be packed with large brown granules which were presumably either entirely or partly composed of vitamin C. The typical appearance of one of these cells is shown in Fig. 29.

Skin and Hair.

The skin appears to contain relatively large amounts of vitamin C; the component cells certainly showed numerous argentophile granules. In the stratum germinativum eighteen or twenty large distinct granules were common in the cells, and in some a few batonettes occurred; in some cells these batonettes were associated with the membrane of the cell, and were orientated with their long axes parallel to the surface of the cell. In the stratum granulosum some of the cells showed quite a large number of small granules, some eighty or ninety. Some of the cells in this region were packed with fatty droplets—these were the cells of sebaceous glands. Vitamin C granules were present in these cells also, and were distributed between the droplets without showing any intimate association with them.

In the stratum lucidum the cells were flattened and contained rather fewer granules of vitamin C, and the granules were much smaller than in the cells of the neighbouring zones.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The cells of the stratum corneum contained no vitamin C granules since the vitamin had probably been completely oxidised.

Some of the subcutaneous connective tissue cells showed thirty or forty large rounded granules, and in a few cells batonettes were also present.

In a section through the base of a hair it was found that the cells constituting the outer root sheath contained a large number of argentophile granules; the cells of Henle's and Huxley's layers, however, contained no granules at all. The cells of the hair papilla possessed a number of fine argentophile granules (Figs. 56-63).

Vitamin C in the Ovary.

Leblond and his colleagues have given some description of vitamin C in the ovary and corpus luteum. Gough and Zilva (1933) were the first to conduct staining tests for vitamin C on the ovary, and found a blackening with silver nitrate. They found, however, that the corpus luteum blackened considerably more than the main mass of the ovary.

In the present work no trace of vitamin C could be found in the cells of the membrana granulosa, the discus proligerus, or the zona pellucida, but a number of fine granules were found encircling the ovum near the membrane, and a few were scattered in the cytoplasm of the egg between the fatty granules. The stroma cells of the ovary reacted very feebly, showing only one or two granules, but the occasional groups of interstitial cells, which appeared to be most common in the region of the follicle, contained quite a number of granules.

As many as thirty large round granules were found in some of the cells; in others, however, only twelve or so were observed; the distribution within the cell showed no special features, the distribution of the granules being apparently indiscriminate (Figs. 67-69).

DISTRIBUTION OF VITAMIN C IN TISSUES HAVING A HIGH CONCENTRATION.

The three organs containing the greatest amount of the vitamin are the adrenal, the pituitary glands, and the corpus luteum.

Figures showing distribution of Vitamin C granules in the cells of various tissues. The large granules represent the vitamin.

Figures 56 and 57. Skin. Cells from stratum germinativum. **Figure 58.** Skin. Cells from stratum granulosum. **Figure 59.** Skin. Cell from sebaceous gland. **Figure 60.** Subcutaneous conective tissue cell. **Figure 61.** Skin. Cell from stratum lucidum. **Figure 62.** T.S. Skin, showing vitamin C granules in practically all cells. **Figure 63.** T.S. developing hair follicle: **A.** Outer root sheath, with vitamin C granules; **B and C.** Inner root sheath: B. Henle's layer, C. Huxley's layer, No vitamin granules; **D.** Epithelial cells of developing hair. With vitamin C granules. **Figure 64.** Ovum in follicle: **A.** Membrana granulosa; no granules of vitamin C; **B.** Discus proligerus; no granules of vitamin C; **C.** Zona pellucida; **D.** Ovum, with a few vitamin C granules situated peripherally; **E.** Nucleus of ovum with nucleoli; **F.** Yolk globule; **G.** Group of interstitial cells with vitamin C granules. **Figures 65–69.** Ovarian interstitial cells showing vitamin C granules.

Leblond has investigated these three. But the present work is restricted to the anterior pituitary gland and the adrenal.

Adrenal.

The distribution of vitamin C in the adrenal has been the subject of previous papers.

Leblond considers that the present author, Siehrs and Miller (1932), and Moore and Ray (1932), are incorrect in describing a positive reaction of the adrenal medulla to the silver nitrate test. In previous papers it has been shown that following a defence reaction of the adrenal medulla, this region of the gland

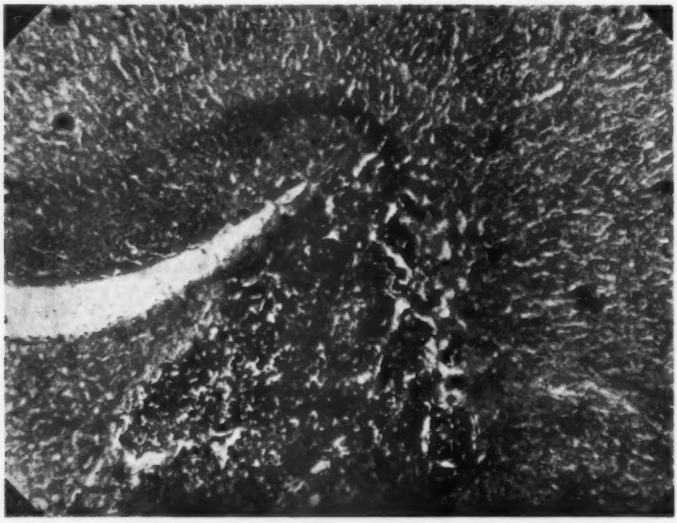
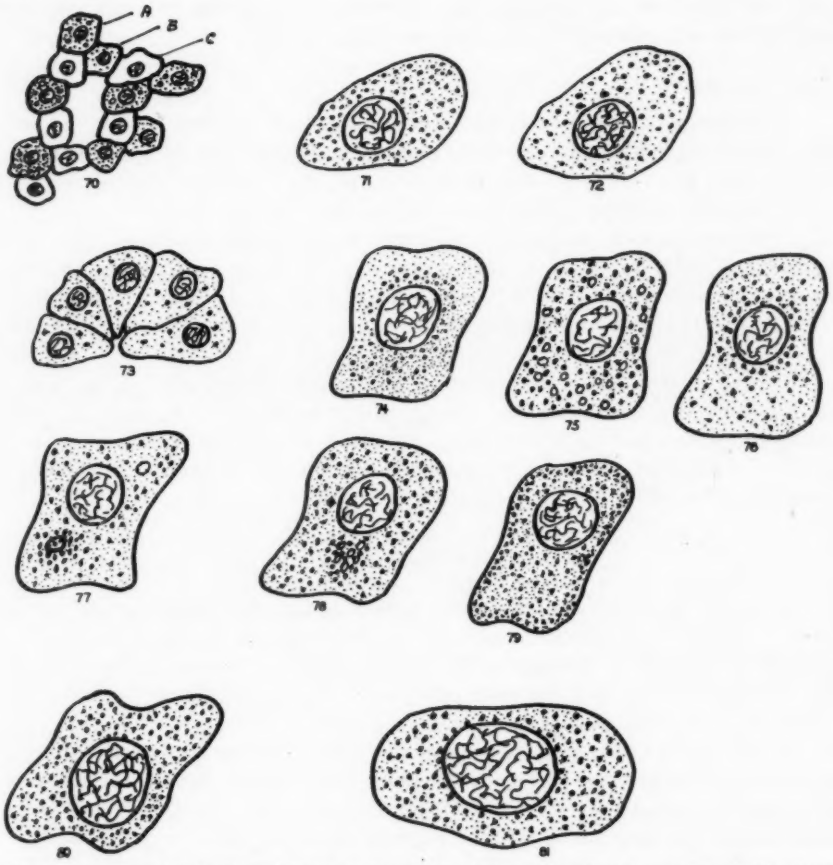


Figure 82. Photomicrograph of medulla and cortex of adrenal gland to show intense blackening of medulla with silver nitrate after ether anaesthesia, indicating that the reversibily oxidized vitamin C normally present in the medullary cells has been reduced owing to the medulla having given a defence reaction.

does give the silver nitrate reaction. Harris and Ray (1933) have shown that vitamin C exists in the adrenal medulla in a reversibly oxidized state in which it does not react with silver nitrate. Apparently then, as has been recently pointed out, the reversibly oxidized vitamin C in the medulla becomes changed to the reducing state if the adrenal medulla is stimulated, particularly by chloroform or ether anaesthesia. The cells in such cases show a very dense deposition of black granules through the cytoplasm, and in some cases immediately surrounding the nucleus. (See Figs. 80, 81, and 82).

Leblond also contends that the zona glomerulosa contains no vitamin C. In all my preparations the zona glomerulosa cells contain a few granules of the vitamin, though they admittedly contain less than the main body of the cortex (Fig. 73).



Figures showing distribution of Vitamin C granules in the cells of various tissues. The large granules represent the vitamin.

Figure 70. Group of cells from anterior pituitary showing: **A.** Chromophil cell containing droplets of lipochrome pigment and large numbers of granules of vitamin C; **B.** Chromophil cell, without lipochrome droplets and with fewer granules of vitamin C; **C.** Chromophobe cell containing no pigment and no vitamin C; **Figures 71 and 72.** Chromophil cells of the anterior pituitary under higher power; no droplets of lipochrome shown. **Figure 73.** Adrenal gland, cells of zona glomerulosa. **Figures 74–79.** Adrenal gland cells of zona fasciculata with and without lipoid droplets. **Figures 80 and 81.** Cells of adrenal medulla after the gland has given a defence reaction; showing a large number of argentophile granules which are probably vitamin C.

Variation in the arrangement of the vitamin C granules was observed in the various zona fasciculata cells. In some there was a tendency towards perinuclear condensation, and in others an aggregation round the lipoid droplets. In some cases there was a condensation around the membrane of the cell. Other cells were observed in which these aggregations did not occur, presumably indicating that the cell was in a different functional state (Figs. 74-79).

The Pituitary.

All authors appear to be in agreement that only the anterior lobe of the pituitary gland stains with any degree of intensity with silver nitrate. In this region Leblond has shown that (in the guinea-pig, rat, etc.) only the chromophil cells of the anterior pituitary gland react with the silver nitrate.

The present work on the fox pituitary confirms this statement. In these chromophil cells in the fox, there were contained many droplets of lipochrome pigment, and in those cells in which the pigment was most common there was the greatest number of granules of vitamin C. Most of the chromophobe cells appeared to contain a few granules of vitamin C, but these were only two or three in number. The disposition of the granules within the cell was not in the direction of a perinuclear or a peripheral concentration. In some cases the nucleus was clear but the surrounding cytoplasm was packed with the granules, while in others the nucleus was completely surrounded by granules which appeared to be in contact with the nucleus (Figs. 70, 71, and 72).

DISCUSSION.

From the present work and that of Leblond and other Paris workers, it appears that almost every cell in the body can be shown to contain at least a few granules of vitamin C. It may be that cells, in higher mammals at least, require the presence of vitamin C for their existence. One can thus easily conceive the reason for such a general tissue breakdown as occurs in acute scurvy.

In the ability of the vitamin to form an oxidation reduction system with glutathione lies probably the secret of its value. Leblond and the Paris school consider that its association with oxidations and reductions in the cell makes the possibility of its function in cellular respiration quite a likely hypothesis. The universality of the distribution of the vitamin in the tissues and organs of mammals adds even more likelihood to this suggestion.

One of the most striking things about the appearance of the vitamin C in the cells of various organs and tissues is its remarkable resemblance in some cases to mitochondria, a fact upon which Leblond has also commented.

The high concentration of the vitamin in certain of the endocrine organs hardly indicates a high rate of respiration in the cells, but it certainly suggests, when coupled with a study of the distribution of these granules in the cell, the

relationship of the vitamin with certain cellular syntheses. It is of no small interest that all those cells which contain a high concentration of substances, such as lipochrome pigments, lipoids and fats, cholesterol and its esters, contain also large quantities of vitamin C. In the face of the present evidence it is suggested that vitamin C is associated non-specifically with the production of the hormones of the adrenal cortex and medulla, the anterior pituitary gland, the corpus luteum, and the interstitial cells of the gonads.

SUMMARY.

An account has been given of the form, distribution and relative amounts of vitamin C in the various organs and tissues of the body of the fox (*Vulpes vulpes*).

Almost every type of cell in the body has been shown to contain some vitamin C, and in view of this fact Leblond's suggestion that vitamin C may be associated with cell respiration is supported.

The high concentration of vitamin C in the cells of the anterior lobe of the pituitary gland, the adrenal, the corpus luteum, and the interstitial cells of the gonads has led to the suggestion that vitamin C is intimately associated with the production of the hormones of these organs.

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NOTES ON THE OCCURRENCE OF AZOTOBACTER IN SOME SOUTH AUSTRALIAN SOILS

by

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Although the distribution of *Azotobacter* in other parts of the world has received considerable attention, there is only one paper referring to its occurrence in South Australia. H. K. Lewcock (1925) published a paper dealing with the stimulating effects of phosphates on nitrogen fixation, and in it he refers to unpublished work which showed the "universal distribution of a vigorous *Azotobacter* flora in South Australian soils . . . . ranging in type from a light calcareous sand, carrying virgin mallee scrub, to a heavy, black, alluvial, truck soil. In every instance also a dense *Azotobacter* film was formed in Ashby mannite media within 6 days . . . ". Unfortunately the details of these experiments are no longer available.

During 1934 the writer carried out an investigation of the occurrence of *Azotobacter* in a number of South Australian soils, and the observations made are offered as a contribution to our limited knowledge of this subject as far as Australia is concerned.

Lewcock utilized Ashby's liquid culture medium for his investigations, but the more recent development of Winogradsky's (1926) silica-gel plate method allows a more satisfactory technique to be employed. This method consists of innoculating finely divided soil on to a silica-gel plate impregnated with a suitable nitrogen-free culture medium, and by counting the colonies appearing on the plate, a quantitative estimation may be made of the number of Azotobacter colonies occurring in the soil. The gels for this method were prepared in 5-inch petri dishes. The amount of soil used varied with the Azotobacter content, a trial count being usually done with a 3 inch dish in order to obtain some idea of the quantity required to give a reasonable number of colonies on the plate; with soils of very low Azotobacter content up to 2 gm. of soil were used. The dish was kept at 30°C., and counts made daily for 7 days.

Although the method is described as quantitative, it is only roughly so, and the counts on any given area of constant chemical and botanical features will depend on the depths at which samples were taken, the time of the year, climatic conditions of existing and past seasons, and the degree of fineness of the sample used. The last condition is rather important (Batchelor (1933), Curie (1931)), and the writer used samples which had been passed through a 30-mesh sieve. At

least two counts have been done on each sample; with *Azotobacter* colonies the agreement is quite good (the difference is usually less than 20 p.c.), but with the small type of colony the error may be very much larger. Counts have been done on 33 soils from various parts of the State, and the results together with the details of the soils under consideration are given in Table 1.

Colonies of unidentified organisms, easily distinguished from *Azotobacter* by their extreme smallness, appeared on the plates with some of the samples, and the counts obtained are also given in the table. These colonies varied in size from about 1 mm. to 0·5 mm. diameter or even smaller, and were best observed on fairly moist (but not wet) gels. Microscopic examination was difficult on account of the size and rigidity of the colonies, but no *Azotobacter* cells were observed, and probably several types of organisms are included in the group. They all occurred in barren soils, and had an incubation period (at 30°C.) of three days or longer. Very little work has been done on these organisms, but it may be mentioned that the colonies from No. 30 produced vigorous but heterogenous growth on mannite-agar slants, but no growth on Ashby's liquid mannite media.

TABLE 1.

No.	Locality.	When sample taken.	Age of sample when count done.	Count per gram. Azotobacter.	Count per gram. Other species.	Growth on Ashby's medium.	pH	Remarks.
			ADELAIDE PLAINS.					
1.	Lockleys	Jan.	8 months	0	6	—	6·3	Sandy soil.
2.	West Mitcham	Aug.	1 month	138	0	Strong white	7·1	Originally derived from soil similar to 3. Under cultivation for past 10 years.
3.	West Mitcham	Feb.	5 months	0	110	Very slight film	6·2	Red-brown earth. Grazed annually. No cultivation for at least 25 years.
4.	Dublin	Jan.	7 months	45	0	—	7·2	Sandy soil used for wheat.
12.	Findon	March	3 months	560	0	Strong white	7·0	P_2O_5 ·30%.
13.	Findon	March	8 months	7	0	—	6·9	P_2O_5 ·15%.
14.	Findon	March	4 months	18	0	—	7·0	P_2O_5 ·14%.
17.	Direk Siding	April	5 months	0	0	—	6·2	Fallow land.
27.	Oaklands	May	2 months	0	0	—	5·9	From a vineyard.
47.	Marino	Sept.	2 months	0	0	—	7·4	Calcareous soil taken about 100 yards S.W. of railway station.
			MOUNT LOFTY RANGES.					
33.	Littlehampton	July	3 months	0	0	—	5·0	Subterranean clover land under sheep.
48.	Eden	Nov.	1 month	0	0	—	6·3	Fairly rich soil from side of gully.
			SOUTH EAST.					
20.	Mount Gambier	April	5 months	0	0	Very slight film	5·0	Sandy soil.
28.	Near Lake Leake	July	3 weeks	0	0	—	5·7	Sandy soil P_2O_5 ·14%.

No.	Locality.	When sample taken.	Age of sample when count done.	Count per gram. Azotobacter.	Other species.	Growth on Ashby's medium.	pH	Remarks.
				MURRAY LANDS.				
49.	Wanbi	Dec.	1 week	0	0	—	7·0	Land under wheat, sand.
50.	Wanbi	Dec.	1 week	0	0	—	7·0	Fallow land, sand.
				YORKE PENINSULA.				
9.	Corny Point	Feb.	5 months	0	0	—	7·1	Manganese deficient.
				MID NORTH.				
6.	Appila	Jan.	7 months	0	2	Very slight film	7·3	Heavy red clay.
29.	Quorn	July	3 weeks	0	0	—	7·8	Red sand. Wheat land fallow 2–3 miles north of town.
31.	Seven Hills	July	3 weeks	0	0	—	6·2	Sandy Eucalyptus and Acacia scrub land. 1 mile south of town.
				FAR NORTH.				
30.	Wilpena Pound	July	2 months	0	33	Very slight film	6·7	Euc. rostrata and grass country just inside the pound.
32.	Hawker	July	1 month	0	0	—	7·7	Approx. ¼ mile N.W. of railway station. Wheat crop just up.
36.	Blinman	July	2 months	0	0	—	7·3	Just north of town before mines.
37.	Ferguson's Gorge	July	3 months	0·4	0	Slight film	7·4	About 2 miles west of Angorichina Hostel, from side of creek.
38.	Koonamore	Aug.	2 weeks	0	0	—	7·7	University reserve. Silt flat carrying annual herbage.
39.	Koonamore	Aug.	1 month	0	26	Very slight film	7·3	Sand hill carrying mulga and annual herbage.
40.	Koonamore	Aug.	2 months	0	0	—	7·7	Salt bush soil.
41.	Pandi	Aug.	1 month	4	16	Strong white	7·0	Flood plain adjacent to Diamantina.
42.	Pandi	Aug.	2 months	0	0	—	7·0	Sand hill on Diamantina flood plain.
43.	Karatunka	Aug.	1 month	26	7	—	7·1	On Diamantina, 35 miles from Birdsville. Sandhill.
44.	Diamantina Flood Plain	Aug.	3 weeks	52	0	—	7·0	From along cattle creek.
45.	Gibber Plain	Aug.	1 month	2	3	—	7·0	At 7-mile bore.
46.	Clayton Bore	Aug.	6 weeks	0	0	—	above 8	Highly saline.

Two types of *Azotobacter* colonies were observed, white, raised colonies (the "colonies seches" of Winogradsky) and the flat, more fluid type ("colonies fluides"), the distribution of the two types being apparently quite haphazard. The growth in the case of No. 37 was much more flat and watery than the usual "fluid colony" type. The growths from all soils containing *Azotobacter* have been examined microscopically, using either gentian-violet (Winogradsky, 1926) or carbol-fuschin as staining agent. Although there was some variation in form,

all showed the typical coccus or oval form, 1·8 to 3·0μ long and 1·2 to 1·8μ broad, diplococci and tetrads being common.

As no soils have been obtained from many important areas, no statements can be made concerning the distribution of *Azotobacter* in this state, but the results show that Lewcock's claim for a universal distribution cannot be substantiated. Further, it seems probable that even in the majority of soils containing *Azotobacter,* the numbers present are so small that their effect on the nitrogen cycle will be almost negligible. Reasons for the absence of *Azotobacter* in the soils examined are not obvious. An interesting point is the limited number of areas in which the bacteria have been found, namely the Adelaide Plains, the Diamantina cattle track, and the isolated instance of Ferguson's Gorge, but the most important fact is that only those soils of pH between 6·9 and 7·4 (approximately) contain *Azotobacter;* this is surprising, as workers in other parts of the world find that pH limits are from 6·0 to about 8·0. Chlorides have a toxic effect (Lipman, 1911), but except with No. 46, are probably not responsible. Two other factors which may be of importance are lack of sufficient carbohydrate material and inadequate aeration of the soil in its natural state. The low phosphate content of South Australian soils by itself will probably have no effect on their distribution, as it has been shown by Greene (1933) that in Arizona the *Azotobacter* flora have adapted themselves to somewhat similar conditions.

In view of the discrepancy between these results and those of Lewcock, a number of soils have been tested in Ashby's liquid media, the results being included in Table 1. In the cases where a "very slight film" is recorded, the result was due to anaerobic action at the bottom of the flask. This film, which only appeared in 6 to 10 days, was extremely thin and of a dirty brown colour, and could not possibly be confused with the result given by Nos. 2, 12, and 14, which gave a definite white *Azotobacter* film in 4 to 5 days.

One quantitative estimation on the nitrogen fixed in liquid media was carried out on No. 12 using 2 gm. mannite, 1 gm. calcium carbonate, 10 gm. soil, and 100 ml. of Ashby's solution. Two flasks (with blanks) were incubated; 0·02 gm. potassium phosphate was added to the first flask, and the second contained no phosphate apart from that introduced by the soil sample. After 7 days' incubation at 30°C., the film on the first flask was jet black, while that on the second was orange brown and much thinner. The nitrogen fixed per gm. of mannite for the flask containing potassium phosphate was 8·5 mg., and for the other flask 5·0 mg.

SUMMARY.

Thirty-three South Australian soils have been examined for *Azotobacter* content by Winogradsky's silica-gel plate method. Only ten soils were found to contain *Azotobacter,* the average count for the active soils being 85 colonies per gm., with a maximum count of 560 per gm.

As far as can be ascertained the only factor governing the distribution of *Azotobacter* is the soil reaction, the pH of all the active soils lying between 6·9 and 7·4.

ACKNOWLEDGMENTS.

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